Physiology and Molecular Biology of Stress Tolerance in Plants

Edited By
K. V. Madhava Rao
A. S. Raghavendra
K. Janardhan Reddy

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PHYSIOLOGY AND MOLECULAR BIOLOGY OF STRESS TOLERANCE IN PLANTS

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Statice (*Limonium latifolium*) plants are utilized as a model to understand metabolic adaptations to environmental stress. Synthetic pathway to the osmoprotectant beta-alanine betaine was discovered in this species and cDNA for beta-alanine N-methyltransferase involved in this pathway is utilized for metabolic engineering of crops for enhanced tolerance to salinity and drought (See Chapter 9).

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WE THANK

Mrs Syamala Madhava Rao

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PREFACE

Increasing agricultural productivity to meet the demands of growing population is a challenging task. Abiotic stresses are among the major limiting factors on agriculture. The knowledge and research programmes on the physiology and molecular biology of stress tolerance are certainly helpful to counter act this negative effect to a great extent. The present literature deals in detail mostly with plant responses to different abiotic stresses. There have been extensive studies, in the past few decades, on the physiology and biochemistry of plant responses to abiotic stress conditions, in the laboratory as well as in the field. However, the interest has shifted to molecular biology of stress tolerance, modes of installing tolerance mechanisms in crop plants. Microarray technology, functional genomics, development of high throughput proteomics would benefit and guide the physiologists, molecular biologists and biotechnologists to enhance stress tolerance in plants. We therefore, felt very strongly that there is an immediate and urgent need for a textbook on this important topic.

This book would be an ideal source of scientific information to the postgraduate students, research workers, faculty and scientists involved in agriculture, plant sciences, molecular biology, biochemistry, biotechnology and related areas.

We would like to thank the authors for their interest and cooperation in this exciting venture. We are grateful to Jacco Flipsen and Noeline Gibson of Springer for their continuous support and technical advice in bringing out the book.

K.V. Madhava Rao
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CHAPTER 1

INTRODUCTION

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Keywords: *Abiotic stresses, functional genomics, genetic engineering, gene products, gene transfer, signal transduction*

Higher plants are sessile and therefore cannot escape from abiotic stress factors. They are continuously exposed to different abiotic stress factors without any protection. On the other hand animals are mobile and can escape the direct harsh conditions. The immobile nature of plants needs more protection. This enabled them to develop unique molecular mechanisms to cope with different stress factors. However, variations do exist in tolerance mechanisms among plants. Certain morphological features of some plants however, make them avoid stress factors. But it may not be the case in all plants. The only option for plants is to alter their physiologies, metabolic mechanisms, gene expressions and developmental activities to cope with the stress effects. Therefore, plants possess unique and sophisticated mechanisms to tolerate abiotic stresses. Those plants that have better tolerant, resistant, protective and acclimation mechanisms alone can survive while others cannot. Gene products play a key role in the molecular mechanisms of stress tolerance in plants.

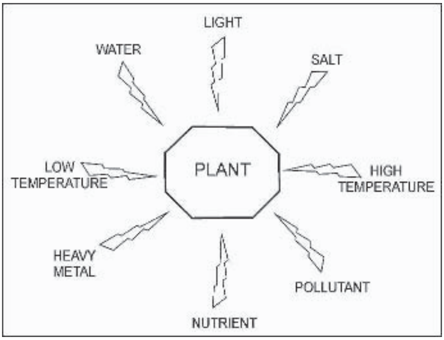

Figure 1. *Some common abiotic stress factors that affect plants*

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Abiotic stresses are commonly caused by drought, salinity, high or low temperatures, light, deficient or excess nutrients, heavy metals, pollutants etc either individually or in combinations (Figure 1). The stress caused by abiotic factors alter plant metabolism leading to negative effects on growth, development and productivity of plants (Figure 2). If the stress become harsh and/or continues for longer period it may lead to unbearable metabolic burden on cells leading to reduced growth and in extreme cases results in plant death. However, plant stress may vary from zero to severe through mild and moderate levels. In nature, plants may not be totally free from stresses. Plants are expected to experience some degree of stress of any factor or factors. To combat these stresses, plants exhibit several mechanisms which make them withstand the stress with the formation of new molecules and molecular mechanisms of stress tolerance.

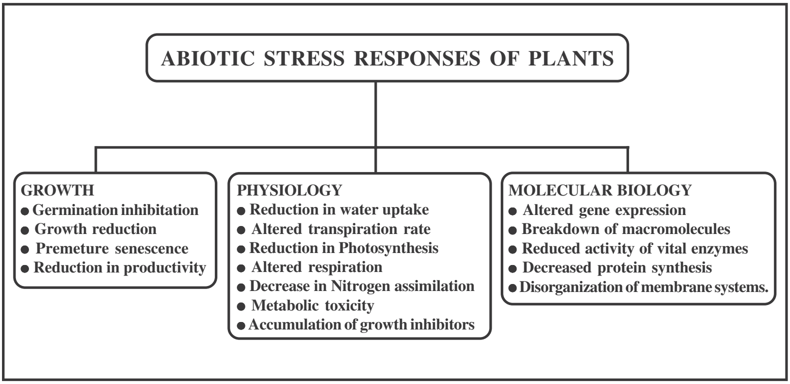

Figure 2. *Some of the common plant responces to abiotic stresses*

Avoidance mechanisms though considered to be advanced, which by modification of morpholgy and anatomy prevents plants from various stress factors, they may not be of much importantce in immediate crop improvement. Therefore, the immediate emphasis is on the development of tolerance mechanisms in plants, since plants exhibit great variations in their tolerance mechanisms, within species, between species and among the plants of different groups. These variations are highly significant in developing stress tolarence in plants.

1. THE MOLECULAR "CROSS ROADS"

Most of the stress factors produce certain common effects on plants although each stress factor has got its own specific effects. The common targets of most of abiotic stress factors are the membrane systems, which under normal conditions perfom several life maintenace processes (Figure 3.). Therefore, all membrane involving processes will be affected by abiotic stresses. Active oxygen species (AOS) are always associated with aerobic life (Vranova et al., 2002). Abiotic stresses such as water stress, salt stresss, temperature stress, light stress, nutrient stress, heavy metal stress and pollution stress are known to accelerate the production of AOS in plants that cause damage to membrane systems and other cellular processes (Dat et al., 2000; Mittler, 2002; Mittler et al., 2004). Antioxidative systems, both enzymatic and nonenzymatic systems, play an important

- ⋆ **Boundary Layer of cells and organelles**
- ⋆ **Metabolic compartmentations**
- ⋆ **Electron transport**
- ⋆ **Signal perception**
- ⋆ **ATP generation**
- ⋆ **Solute transport**
- ⋆ **Ion pumps**
- ⋆ **Ion channels**
- ⋆ **Carrier proteins**
- ⋆ **Electrical excitability**
- ⋆ **Anchoring points for proteins**

Figure 3. *Certain functions of plant membrane systems*

role in balancing and preventing oxidative damage (Bowler et al., 1994; Foyer et al., 1994). However, the prodction and efficiency of the antioxidative systems depend on plant type and genetic make up of the plant. In spite of the close association of AOS with aerobic life, their production, role, stress involvement, importance in signaling phenomena and their scavenging are not clearly elucidated. In addition, abiotic stresses

SOME OF THE PROMINENT ABIOTIC STRESS TOLERANCE MECHANISMS

- Activation of signaling factors
- Altered gene expression
- Accumulation of compatible solutes
- Synthesis of stress proteins
- Enhanced antioxidative metabolism
- Ion homeostasis and compartmentation
- Facilitated membrane transport
- Accumulation of polyamines
- Adjustment of hormonal balance

Figure 4. *Some of the prominent abiotic stress tolerance mechanisms*

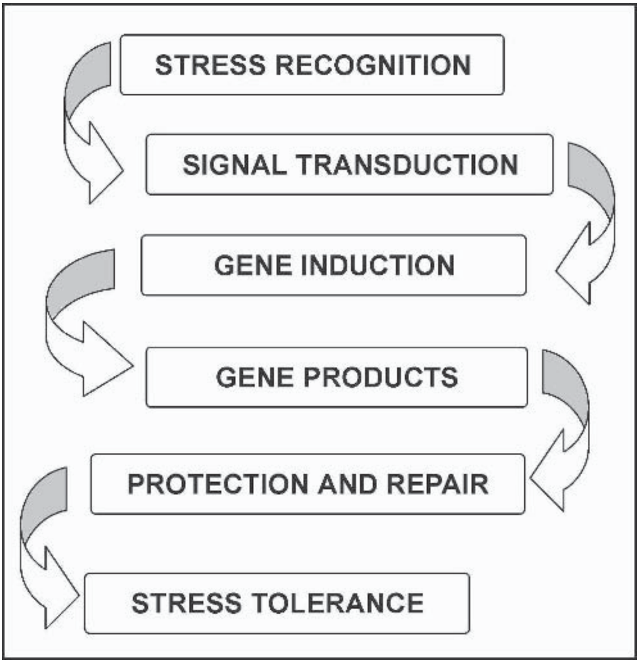

Figure 5. *The path of stress tolerance in plants*

affect photosynthesis, respiration, nitrogen assimilation, protein synthesis and several other processes (Figure 2). To combat stress effects plants develop some common tolerance mechanisms as well as stressor specific mechanisms to cope up with stress (Figure 4). However, the degree of tolerance varies from plant to plant, from low to high. Stress tolerance mechanisms start with stress perception followed by the formation of gene products that are involved in cellular protection and repair (Figure 5). The signal transduction pathways that detect stress play a crucial role in the induction of stress tolerance in plants (Smalle and Vierstra, 2004). One of the important ways to develop stress tolerance is by gene transfer (Figure 6).

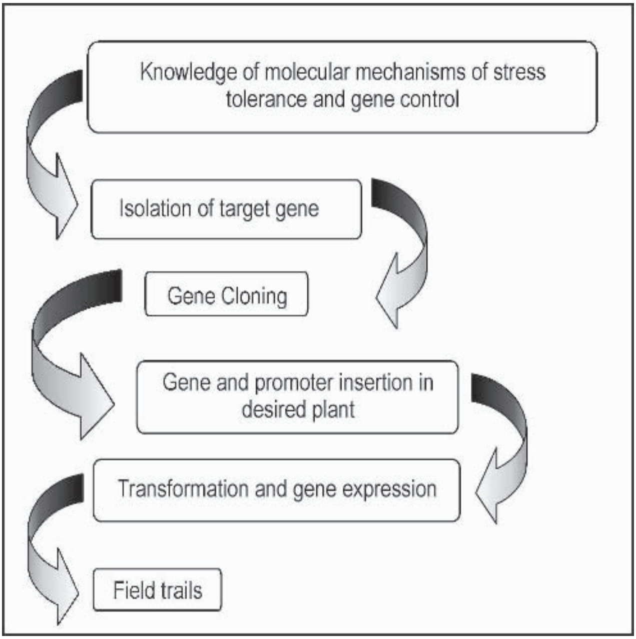

Figure 6. *Strategies of gene transfer in plants*

This book attempts to present an overview on the physiology and molecular biology of plant tolerance mechanisms in response to most important abiotic stress factors. The present chapter describes the scope of the articles included in this book. There have been some books published earlier on this topic (Jones et al., 1989; Fowden et al., 1993; McKersie and Leshem, 1994; Basra, 1994; Basra and Basra, 1997; Pessarakli, 1999; Cherry et al., 2000; Hirt and Shinozaki, 2002; Di Toppi and Pawlik-Skowronska, 2003; Ashraf and Harris, 2005; Jenks and Hasegawa, 2005; Chakraborty and Chakraborthy, 2005). Some of these books may either deal with physiology or molecular biology, but none on physiology and molecular biology together.

2. WATER STRESS

Drought leading to water stress in plants is a major problem in reducing agricultural productivity especially in tropical, semi-arid and arid regions of the world. Water deficits result from low and eratic rain fall, poor soil water storage and when the rate of transpiration exceeds water uptake by plants. The cellular water deficits results in the concentration of solutes, loss of turgor, change in cell volume, disruption of water potential gradients, change in membrane integrity, denaturation of proteins and several physiological and molecular components (Grifth and Parry, 2002; Lawlor 2002; Lawlor and Cornic, 2002; Raymond and Smirnoff, 2002: Parry et al., 2002; Bartels and Souer, 2003). The stress effects depend on the degree and duration of the stress, developmental stage of the plant, genotypic capacity of species and environmental interactions. Several attempts were made to understand the water stress recognition and the subsequent signal transduction (Bohnert et al., 1995; Leung and Giraudat, 1998). The gene induction leads to the formation of gene products such as proline, glycinebetaine, and other products, which may act to maintain cellular function through protection of cellular processes by protection of cellular structures and osmotic adjustments (Bray, 1993; 1997; 2002). Abscisic acid concentration increases under water stress as well as under some other abiotic stresses (Christmann et al., 2005). In fact abscisic acid is considered as a 'stress hormone' (Zeevaart and Creelman, 1988) although it may serve several other functions in the absence of stress. Understanding the functions of the various gene products formed, which are usually involved in osmotic adjustment, protection and repair of cellular structures, are of great value in evaluating water stress tolerance mechanisms and to develop water stress tolerant plants. A large number of genes with a potential role in water stress tolerance have been identified and characterized (Ingram and Bartels, 1996). In spite of the considerable progress made in understanding plant molecular responses to water deficits and its impact on whole-plant physiology, the details of water stress perception; signal transduction and molecular biology of water stress tolerance are yet to be evaluated (Chapter 2, Yakota, Takahara and Akashi).

3. SALT STRESS

Salinity affects agricultural production and its quality in arid and semiarid regions, where rainfall is limited and is not sufficient to transport salts from the plant root zone (Quesada et al., 2000; Tester and Davenport, 2003). Poor water management also results in salinity. The basis for salinity is evaporation in which water evaporates in a pure state leaving salts and other substances behind (Carter, 1975). Salinity arises due to increase in the concentration of salts like sodium chloride, sodium carbonate, sodium sulphate or salts of magnesium. The dominant salts are either sodium chloride or sodium sulphate or mixtures of the two. The saline soil management includes crop selection, crop stand establishment, leaching requirement, drainage and other reclamation practices. It is also anticipated that the importance of salinity as a breeding objective will increase in

the future (Flowers and Yeo, 1995). The effect of salinity on plants is complex and its adverse effects include ion toxicity, water deficits and nutrient imbalance and deficiencies. Much information is available on morphological and anatomical adaptations in response to salinity (Poljakof-Mayber, 1975). Considerable information on physiological and molecular responses of plants to salinity stress is also available (Adams et al., 1992; Moons et al., 1995; Hasegawa et al., 2000; Munn, 2002; Zörb et al., 2005). Salt tolerance and resistance mechanisms are highly complex since the effects are diverse and are controlled by a number of genes or groups of genes (Flowers and Yeo, 1995). Salt tolerance is generally associated with regulated ion uptake, compartmentation of ions and gene products including stress proteins (Flowers and Yeo, 1986; Cheeseman, 1988; Winicov, 1998; Zhu, 2001). Ion homeostasis is an important component of salt tolerance (Zhu, 2002). However, based upon its complexicity, the mechanisms underlying salt tolerance are to be investigated in detail. Dajic in Chapter 3 deals in detail the molecular basis of salt tolerance in addition to related physiological, genetical and biotechnological aspects.

4. HIGH TEMPERATURE STRESS

High temperature stress in plants arises in response to many factors such as the exposure of plants to high ambient temperatures, exposure of germinating seeds to the soil which is warmed by absorbed infrared radiation from the sun, more plant transpiration followed by less water absorption, reduced transpiration capacity in certain plant organs, forest fires, natural gas blowouts, etc. Though, much work has been carried out at ultrastructural, molecular and gene expression level under different temperature extremes, the temperature perception and the molecules involved in the perception are not known clearly (Burke and Usda-Ars, 1988; Iba, 2002; Rao et al., 2002; Camejo et al., 2005). All the cells of an organism respond to high temperature stress. All organisms when exposed to rapid increases in external temperatures, generally 5 to 10 ^{0}C above normal growth temperatures for a period of few minutes to a few hours exhibit synthesis of an elite set of proteins called heat shock proteins (HSPs) which are not present, or are present in small quantities in unstressed organisms (Sridevi et al., 1999). These HSPs are involved in cellular repair, rescue, cleanup and/or protection during the stress and from its recovery. Understanding the mechanisms and development of thermotolerant plants is of great significance in tropical, semi-arid and arid regions of the world. Sharkey and Schrader (Chapter 4) emphasizes the effect of high temperature stress on various physiological and molecular biological processes and discusses several strategies for improving heat tolerance in plants.

5. FREEZING STRESS

Plants growing in temperate and frigid areas are exposed to freezing temperatures. It is well known that membrane systems are the primary sites of freezing injury to plants (Rudolf and Crowe, 1985; Hughes and Dunn, 1996; Thomashow, 1999). In addition, cell damage in response to freezing stress is also caused by protein denaturation. Freezing tolerance is characterised by changes in metabolite levels and enzyme activities (Levitt, 1980; Mazur, 1968; Steponkus, 1984; Guy, 1990). Freezing tolerance is associated with the accumulation of sugars, several types of proteins including heat shock proteins, lipids, abscisic acid and other products of altered metabolism (Siminovitch et al., 1968; Nagao et al., 2005). They are expected to depress the freezing point of the tissue, may act as nutrient and energy source and play a key role in rectifying the cellular damage caused by freezing stress. Freezing tolerance increases with decreasing water content. Abscisic acid accumulation also increases freezing tolerance. However, much information is not available regarding the freezing injury and tolerance mechanisms against freezing stress. Recently much interest has been shown towards the identification, characterization and functioning of genes with roles in freezing tolerance and the mechanisms involved in low temperature gene regulation and signal transduction (Thomashow, 1999). In this connection, a systems biology approach to study cold acclimation of plants possesses great significance (Chapter 5: Trischuk, Schilling, Wisniewski and Gusta).

6. PHOTOOXIDATIVE STRESS

Among the abiotic stress factors, light stress is one of the important environmental constraints that limit the efficiency of photosynthesis and plant productivity (Foyer and Noctor, 2000; Das, 2004; Reddy et al., 2004). When absorbed light energy exceeds the capacity for light energy utilization in plant photosynthesis, then the photosynthetic efficiency will be reduced due to the formation of AOS, which can damage photosynthetic apparatus and chloroplast components. In order to mitigate the photooxidative stress, plants have developed certain strategies of tolerance mechanisms (Mittler, 2002; Mittler et al., 2004). Understanding how plants respond to light stress has a high priority in several plant biotechnological programmes. Foyer et al., (1994) and Apel and Hirt, (2004) reviewed the mechanism of photooxidative stress tolerance in higher plants. Chapter 6 (Reddy and Raghavendra) covers the recent advances in elucidating the pivotal role of AOS metabolism in response to photooxidative stress, in addition to variuos physiological and molecular strategies of plants to develop tolerance mechanisms under photoinhibitory conditions.

7. NUTRIENT STRESS

Plant growth, development and yield are contributed by 17 essntial elements (Hopkins and Hüner, 2004). Plants may be subjected to nutrient stress due to several factors such as negligence of the farmer leading to nutrient deficiency or excess supply of nutrients because of the farmer's over enthusiasm to obtain more yield, natural deposits or mining processes etc. Nutrient stress and associated metabolic disorders decrease plant growth and yield (Lynch and Brown, 2001). Plant growth and metabolism is also affected by heavy metal and salinity stress. Developing nutrient stress tolerance in crop plants may help to extend agriculture to unexplored harsh and nutrient poor soils (Cobbet, 2000; Clemens, 2001). Plant growth response to low or excess nutrient stress and related remedial measures to improve crop yields are discussed by Reddy (Chapter 7).

8. HEAVY METAL STRESS

Supra-optimal concentrations of heavy metals such as Cd, Pb, Hg, Cu, Zn and Ni affect growth, development and yield of plants (Pahlsson, 1989; Sresty and Rao, 1999). However, Cu, Zn and Ni are essential micronutrients at low concentrations. Heavy metals affect several physiological (Barceló and Poschenrieder, 1990) and metabolic processes (Van Assche and Clijsters, 1990; Hall, 2002; Schützendübel and Polle, 2002). Plants have developed several mechanisms that control and respond to the uptake and accumulation of both essential and nonessential heavy metals (Cobbet and Goldsbrough, 2002). These tolerance mechanisms in plants vary from species to species and their genetic background. The important heavy metal tolerance mechanisms include, metal binding to wall, reduced transport across the cell membrane, active efflux of metals, compartmentalization, chelation and sequestration of heavy metals by particular ligands such as phytochelatins and metallothioneins (Tomsett and Thurman, 1988; Cobbet and Goldsbrough, 2002). Antioxidative systems are also involved in heavy metal tolerance (Rao and Sresty, 2000). Certain plants specially many brassicaceae family members including numerous Thalspi species have relatively high tolerance for heavy metals such as Ni and Zn and act as hyperaccumulators which can be used for phytoremediation (Clemens, 2001; Freeman et al., 2005). Gasic and Korban (Chapter 8) explore different heavy metal tolerance mechanisms and discuss the importance of hyperaccumulators in phytoremidiation.

9. METABOLIC ENGINEERING

To cope up with different abiotic stresses plants alter their metabolic pathways to adjust to changed environments (Rathinasabapathi, 2000). The metabolic pathways such as proline, glycinebetaine, polyols, antioxidant components become more active to keep the plant survive under stress conditions. However, the initiation and efficiency

of these pathways differ from species to species or genotype to genotype to a great extent. Installing these stress tolerating pathways utilise recombinant DNA technology (Stephanopoulos, 1999). Stitt, (1995) has given an interesting account of production of transgenic plants for metabolic design. The use of novel approaches combining the techniques of genetic, genetic engineering and molecular biology are expected to provide exciting avenues for future research (Madlung and Comai, 2004). Understanding the mechanisms by which plants perceive and transduce stress signals to initiate adaptive response is essential for engineering stress-tolerant crop plants (Xiong and Zhu, 2001). In this direction, various metabolic engineering strategies for stress tolerance in plants is presented by Rathinasabapathi and Kaur in Chapter 9.

10. FUNCTIONAL GENOMICS

Development of techniques such as cDNA libraries, molecular markers, PCR amplifications and microarray technologies made it possible to determine transcript patterns and to identify differentially expressed genes in plants. Comparision of transcript patterns with proteome data may provide information whether the intracellular concentration of specific proteins is preferentially regulated at the level of transcription or by post-transcriptional mechanisms. These techniques help to record the genome wide expression patterns very rapidly and with high accuracy (Kuhn, 2001; Derra, 2004). The information so obtained can be integrated with functional genomic information that contributes to our understanding of the correlation between genes and phenotype of a plant. Based on these techniques, Tyagi, Vij and Saini (Chapter 10) describe the genome-wide approach to develop stress tolerance in plants.

11. PROPELLING FORWARD

Various physiological and molecular mechanisms in association with the applications of plant breeding and genetic engineering can improve the scope for stress tolerance in plants (Figure 7). The present literature on molecular biology deals in detail mostly with abiotic stress tolerance and modes of installing tolerance mechanisms in plants with a view to have desired yields even under harsh environments. The importance given to this line of research is quite evident from the large number of publications appearing on this topic every year. This trend will continue in future. New molecules, their new roles, new concepts and new molecular mechanisms, more attention on products related to stress inducible genes, importance of signal transduction pathways, microarray analyses and functional genomics pervade the field of abiotic stress tolerance. We there-

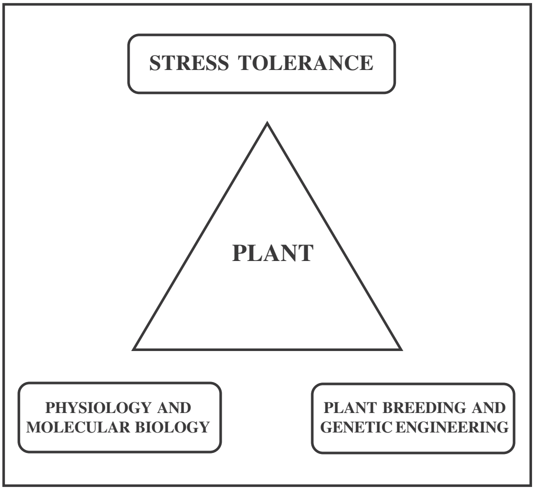

Figure 7. *Knowledge of physiology and molecular biology combined with plant breeding and genetic engineering techniques are expected to enhance stress tolerance in plants*

fore felt very strongly that there is an immediate and urgent need for an advanced level textbook on this important topic. This book would not only review the present status but also trigger further research on this exciting field.

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CHAPTER 2

WATER STRESS

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1. INTRODUCTION

Plants use ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO) to fix CO_2 during photosynthesis. RuBisCO also reacts with O_2, lowering productivity through inevitable photorespiration and increasing the CO_2 compensation point of C_3 plants to 50 to 70 bar. The Km value for CO_2 of RuBisCO is 10 to 15 µM and CO_2 activation is necessary for activity (Roy and Andrews, 2000). Diffusion barriers to CO_2 in the stomata, plasma membranes, cytosolic fluid and chloroplast envelopes lower CO_2 concentrations around RuBisCO to approximately 7 µM during active photosynthesis (von Caemmerer and Evans, 1991; Noctor et al., 2002), even with the aid of aquaporins for quick diffusion at plasma membranes (Terashima and Ono, 2002; Uehlein et al., 2003). Consequently, less than 20% of all RuBisCO catalytic sites actually participate in photosynthetic CO_2 fixation in chloroplasts (McCurry et al., 1981). Nevertheless, plants fix a total of 200 Gtons of CO_2 every year by investing a large amount of nitrogen in RuBisCO synthesis and by maximizing the density of stomata per leaf unit area and the size of stomatal apertures (Terashima et al., 2005).

These properties of RuBisCO are the most critical factors influencing the physiology of plants under water-stressed conditions (Whitney and Andrews, 2001). The amount of water transpired from leaves through stomata is 500 to 1000 times more than the amount of CO_2 absorbed on a molar basis (Larcher, 1995). Consequently, plants need an enormous amount of water for growth. The water use efficiency of C_3 plants is 1.3 to 2 g of dry matter production per kg of water used and this is 2-fold higher in C_4 plants. This indicates the importance of water as a determinant of plant productivity in the field; for example, in the US drought is the most serious environmental stress affecting agricultural production (Table 1) (Boyer, 1982).

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***Table 1**. Distribution of insurance indemnities for crop losses in the United States during the last 40 years (Boyer, 1982)*

Cause of crop loss	*Proportion of payment (%)*
Drought	40.8
Excess water	16.4
Cold	13.8
Hail	11.3
Wind	7.0
Insects	4.5
Disease	2.7
Flood	2.1
Others	1.5

To maximize productivity, plants optimize the morphology, physiology and metabolism of their organs and cells; however, this strategy causes vulnerability to water deficits. To overcome this, plants are equipped with various mechanisms of adaptation to water-limiting environments. The following chapter describes recent advancements in physiological, biochemical and molecular and cellular research related to water deficiency in plants. Although flooding and excess water are other extreme water stresses encountered by plants, and although the inhibitory effect of heavy rainfall on leaf photosynthesis has also been reported (Ishibashi et al., 1996), these topics are not dealt with here.

2. PHYSIOLOGICAL RESPONSES TO DRYING ENVIRONMENT

2.1. Sensing Drying Environments

The absence of precipitation in natural environments causes dryness of the atmosphere and soil, the latter mostly due to evaporation of water from the soil surface in the daytime. In general, drying of soil is slow (Larcher, 1995), but decrease atmospheric humidity can sometimes be quick. Accordingly, plants need suitable systems both in their roots and leaves that sense environmental dryness.

Plant leaves close their stomata immediately on sensing an increase in leaf-air vapor pressure difference, even if the roots have sufficient water (Mott and Parthurst, 1991; Assmann et al., 2000); this response is completed in several minutes (Assmann et al., 2000). Whether this stomatal closure system is abscisic acid (ABA)-dependent or

independent is unknown. Expression of the gene encoding abscisic aldehyde oxidase has been revealed in the guard cells of dehydrated Arabidopsis leaves (Koiwai et al., 2004). Moreover, four other enzymes involved in the ABA-synthetic pathway are known to be expressed in leaves (Iuchi et al., 2001; Tan et al., 2003), but their functional localization remains to be determined. Since exposure of leaves to dry air causes decreases in the turgor of epidermal cells and transpiration rate without any significant effect on the leaf water potential (Shackel and Brinckmann, 1985), the sites of perception of signals of atmospheric dryness and ABA synthesis are thought to be close to or in the guard cells. Although ABA genes are known to be up-regulated under drought conditions, rapid closure of stomata has also been observed in *abi1* and *aba2 Arabidopsis* mutants (Assmann et al., 2000). This is possibly the result of a low basal level of ABA in these mutants, sufficient enough to transmit the leaf-air vapor pressure difference, or indicative of guard cells as the sensor and transducer of humidity signals (Maier-Maercker, 1983).

Evaporation of water lowers the water potential and increases the salt concentration of soil. In general, other stresses such as osmotic and high salt concentration stresses also affect roots in combination with water deficits, while heat stress is a further stress in leaves. This is thought to be reflected by the activation of numerous common factors in inter-/intracellular signal transduction pathways with different environmental stimuli (Yamaguchi-Shinozaki and Shinozaki, 2005). Deficits in the water content of the soil environment might be sensed as an increase in the salt concentration around root surfaces and/or an increase in the osmotic pressure of root cells. However, no water sensor or potential low water sensor has so far been identified in plants. An *Arabidopsis* mutant showing no hydrotropism or directed growth of roots to gradients in moisture has been isolated (Eapen et al., 2005), but the mutated gene(s) remains to be determined.

The ABA is synthesized from carotenoid by ABA-synthesizing enzymes (zeaxanthin epoxidase, 9-*cis*-epoxycarotenoid dioxygenase and aldehyde oxidase) induced in root tip cells or parenchyma cells of vascular bundles by drought and salt stresses (Koiwai et al., 2004). ABA synthesized in the roots enters the xylem vessels in a free form or as a conjugate with glucose, and from here is transported to the leaves (Sauter et al., 2002). How the conjugates are formed in the cytosol of the cortex remains to be determined. The conjugated form is thought to be suitable for long-distance delivery from roots to leaves, since the free form might possibly escape from the acidic xylem sap to surrounding tissues. The ratio of free to conjugated forms of ABA in xylem sap varies from plant to plant, but in all species, the total amount of ABA increases significantly under drought and salt stresses (Sauter et al., 2002).

The ABA conjugates are hydrolyzed into a free form by β-D-glucosidase in the apoplastic space (Dietz et al., 2000), inducing stomatal closure aided by a signaling system in the guard cells (discussed below). The guard cells in the leaves of plants grown under well-irrigated conditions are large in size, while inversely, the stomata of plants grown with limited water are smaller but more dense (more stomata per unit area)

(Elias, 1995). Smaller stomata are advantageous in that the stomatal aperture can be reduced within a short period after guard cells sense ABA. Stomatal closure in many plants is incomplete even after application of high concentrations of ABA (Mustilli et al., 2002). However, field-grown plants, woody plants and wild watermelon plants show almost complete stomatal closure and transpiration rates of almost zero during severe drought stress (Davies et al., 1994; Loewenstein and Pallardy, 1998; Yokota et al., 2002). Since complete stomatal closure cannot be accomplished by application of 300 μM ABA in wild watermelon plants, a possible alternative drought signal from the roots to leaves has been suggested (Yokota et al., 2002).

2.2. Responses of Leaf Photosynthetic Systems to Drying Environments

During progressing drought, plants attempt to protect against evaporation by closing their stomata. However, many plants lose water through stomata that remain open as well as through their cuticles. The water conductance of the cuticle varies greatly from species to species (Kerstiens, 1996); however, the reason for this large variation remains unknown. The lowest conductance value so far reported was with the cuticle of Vanilla plants; this value was much lower than those of artificial food-storage films such as polyvinylchloride and liquid crystal polymer (Kerstiens, 1996; Riederer and Schreiber, 2001). Despite the lack of evidence suggesting a close correlation between cuticle conductance and drought resistance in crops (Kerstiens, 1996), water filled pores of molecular dimension are thought to contribute to cuticular transpiration (Riederer and Schreiber, 2001).

As leaf water is lost, the turgor pressure of leaf tissues decreases and leaves begin to wilt. Wilting or curling of the leaves functions to protect photosynthetic machinery from direct rays of the sun (Larcher, 1995). Since the leaves of some plants such as wild watermelon do not wilt after stomatal closure, they are thought to possess specialized systems able to endure full sunlight virtually in the absence of CO_2 fixation (Kawasaki et al., 2000; Yokota et al., 2002). The morphology of the plant body as well as the molecular and biochemical characteristics of photosynthetic organs has evolved to maximize photon capture and use of these photons in CO_2 fixation. Accordingly, stomatal closure under drought stress deprives plants of their largest consumer of solar energy.

Under non-stressful conditions, half the electrons in plastoquinone enter the Q cycle enabling transportation of more protons to the lumenal side of thylakoids in order to meet the ATP/NADPH ratio required by the photosynthetic carbon reduction (PCR) cycle (Shikanai et al., 2002; Cramer et al., 2004); these electrons are therefore not passed to cytochrome f and consequently photosystem I (PSI). With progressing stomatal closure, the rate of utilization of electrons in PCR and the photorespiratory carbon oxidation (PCO) cycle decreases. Although the rate of oxygen fixation by RuBisCO (photorespiration) increases under these conditions (Cornic and Fresneau, 2002; Noctor et al., 2002), considering the relative specificity of plant RuBisCO and CO_2

and O_2 concentrations in chloroplasts during photosynthesis (Noctor et al., 2002), the rate of energy utilization of photorespiration does not exceed that under non-drought conditions. Electrons in PSI are directed to electron transport chains (Cornic et al., 2000; Golding and Johnson, 2003; Golding et al., 2004) causing oxygen reduction (Asada, 1999; Biehler and Fock, 1996) when utilization of NADPH slows down. Under such conditions, the ATP/ADP ratio increases and the lumenal side of the thylakoids is acidified (Kramer et al., 2004).

In PSII, two carboxyl groups of photosystem II subunit S (PsbS) are protonated and synthesis of zeaxanthin from violaxanthin is promoted at a low luminal pH (Li et al., 2004). Zeaxanthin blocks energy transfer from light-harvesting chlorophylls to the reaction center chlorophyll P680 in PSII (Holt et al., 2004). The energy in light-harvesting chlorophylls is dissipated mainly as heat and partly as fluorescent light, and blocking of energy transfer to P680 or conversion of this energy to heat is detected as non-photochemical quenching (Ma et al., 2003). An increase in non-photochemical quenching is detected in leaves where the supply of photon energy exceeds the demand of RuBisCO-related reactions under drought, strong light and salt stresses (Golding and Johnson, 2003; Teraza et al., 2003). The PSII D1 protein is continuously degraded and replenished during photosynthesis under moderate conditions when no phenotypic damage occurs. The turnover of D1 starts with damaging of grana thylakoids and is completed with the return of the PSII complex replenished with newly synthesized D1 in appressed and stromal thylakoids. Experiments with a cyanobacterium *Synechocystis* sp. PCC6803 have shown inhibition of a translation step in protein synthesis to be the main cause of photoinhibition of PSII at high light intensities (Nishiyama et al., 2004).

Three different routes for the cyclic electron flow around PSI have been suggested. One is through stromal NADPH and plastoquinone (Shikanai et al., 1998a) while another is through ferredoxin and plastoquinone (Munekage et al., 2002). The protein entities of these two routes remain unclear; however, since an Arabidopsis mutant in which the single genes involved in both routes are mutated shows severely suppressed growth (Munekage et al., 2004), both are thought to be essential for normal photosynthesis and likely to function under stresses. The cytochrome b_6f complex isolated from spinach leaves contains a ferredoxin: NADP reductase ratio of 0.9 reductase/1 cytochrome *f* (Zhang and Cramer, 2004), suggesting the existence of the third path in which electrons of NADPH are thought to return to PSI without involvement of plastoquinone.

It is possible that PSI cyclic electron transport increases in momentum under drought stress when solar energy trapped by the thylakoids greatly exceeds the demand from carbon metabolism. PSII is severely down-regulated preventing release of electrons from P680 through acidification of the thylakoid lumen (Golding and Johnson, 2003; Teraza et al., 2003). Since PSI receives photons at a similar frequency to PSII, energy dissipation in PSI should not be neglected. The flux of electrons in PSI cyclic electron transport plays only a minor role relative to the total PSI flux under moderate conditions. In barley leaves suffering CO_2 limitation under drought, the quantum effi-

ciency of PSII decreases greatly, but that of PSI is not influenced (Golding and Johnson, 2003). This observation supports the suggestion that electrons continue to flow in PSI cyclic electron transport chains under such conditions. This mechanism could relieve hyper-excitation of the PSI complex and the flow of electrons to oxygen (Miyake et al., 2005), although it has also been suggested that the oxidized form of P700 ($P700^+$) participates in quenching PSI over-excitation (Owens, 1996; Ort, 2001).

The occurrence of a reduction in oxygen in PSI under moderate conditions is under debate; however, repressed expression of ascorbate peroxidase and a mutation in the ascorbate-synthetic pathway cause severe inhibition of growth in tobacco and *Arabidopsis*, respectively (Orvar and Ellis, 1997; Veljovic-Jovanivic et al., 2001). A reduction in oxygen might also function in the synthesis of surplus ATP during the photosynthetic induction phase (Makino et al., 2002). The transfer of electrons from water in PSII to oxygen in PSI is also thought to play an important role in excess energy dissipation, as the excitation of chlorophyll is so excessive. Superoxide formed in PSI is reduced by thylakoid-bound Cu,Zn-superoxide dismutase to H_2O_2 and then to water by thylakoid-bound ascorbate peroxidase; O_2- and H_2O_2 that escape attack might be decomposed by stromal isoforms of these enzymes (Asada, 1999). Thioredoxin peroxidase, or 2-Cys peroxiredoxin, functions to decompose lipid peroxides (Dietz, 2003).

The above biochemical studies suggest that oxygen reduction in PSI acts as an important electron sink in chloroplasts under stress. However, the genes of the enzymes involved in decomposing active oxygen in the chloroplasts are not up-regulated under drought or salt stresses, unlike the genes for the cytosolic counterparts of these enzymes (Yabuta et al., 2002; 2004). Furthermore, chloroplast APX is a prime target of oxidative stress (Mano et al., 2001). Inactivation of chloroplast APX is much more severe than that of phosphoribulokinase, a light-regulatory SH-enzyme, in tobacco leaves stressed by drought and strong light (Shikanai et al., 1998b). Chloroplast APX is also known to quickly lose its activity *in vitro* in the presence of H_2O_2 (Miyake and Asada, 1996). Local imbalance in the ratio of ascorbate to H_2O_2 in the vicinity of thylakoids might be a determinant of APX activity.

An active flow of electrons to PSI cyclic electron transport chains and oxygen and activation of the malate valve (Fridlyand et al., 1998) induce transfer of protons from the stroma to lumenal side of the thylakoids. However, the thylakoids are not acidified to a level at which the lumenal proteins are denatured. Uncoupling of thylakoid membranes through a change in the stoichiometry of protons transferred in the Q cycle and reduction of the γ-subunit of ATP synthase have been proposed for suppression of hyper acidification of the luminal side of the thylakoids in spinach leaves (Richter et al., 2004). Another example is release of coupling factor 1 from the ATP synthase complex for uncoupling in sunflower plants suffering drought (Teraza et al., 1999).

2.3. The First Sacrifice in Drought

Plants experience a huge difference in light intensity, from that of full sunlight in the daytime to dark at night. Light of high intensity is very damaging to plants under drought conditions, even in the presence of drought tolerance systems. To determine the first drought-induced injury that occurs in plants before complete collapse, Flexas and Medrano (2002) reviewed the literature in an attempt to correlate the biochemical events during drought stress with the physiological parameters (Figure 1). They noticed that the decreases in some biochemical activities corresponded fairly well to decreases in stromatal conductance, but not to decreases in relative water content or mesophyll water potential, during progressing drought. The first biochemical step impaired during drought is ATP synthesis. RuBP regeneration, which is related to this, is also susceptible to mild drought stress. Down-regulation of electron transport occurs under more severe drought conditions, before irreversible PSII damage.

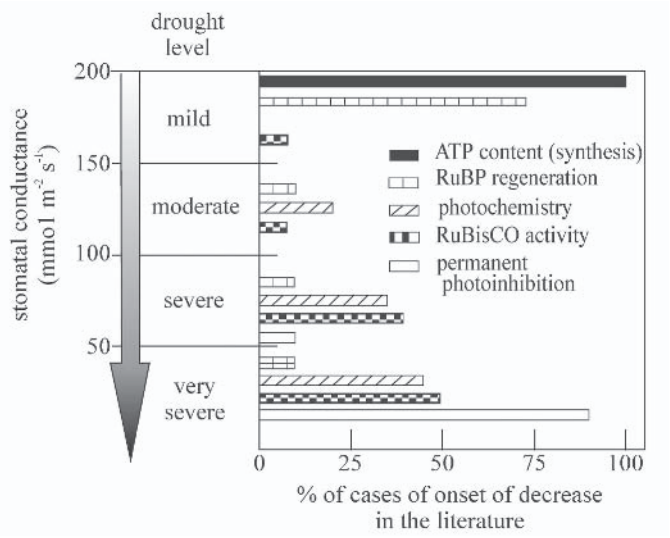

***Figure 1**. Susceptibilities of physiological and metabolic events to drought stress. This figure was adapted from Flexas and Medrano (2002). It shows the degree of stomatal conductance that occurs with each event shown under various drought levels and percentage of the study in which the decrease of the event in question occurred. ATP synthesis was the most susceptible to drought stress*

3. COMPATIBLE SOLUTES AND DROUGHT STRESS

A wide variety of organisms synthesize and accumulate small molecule compounds known as compatible solutes (osmolytes or osmoprotectants) in their cells as a way of

tolerating stresses such as drought, high salt concentrations, and so on. In general, compatible solutes become soluble at high concentrations without inhibition of other cellular components (Ford, 1984). The compatible solutes so far reported in plants include amino acids (proline and citrulline), onium compounds (glycine betaine, 3-dimethylsulfonopropionate), monosaccharide (fructose), sugar alcohols (mannitol and pinitol), and di- and oligo-saccharides (sucrose, trehalose and fructan). Of these, glycine betaine is synthesized in xerophytes and halophytes (Robinson and Jones, 1986), while citrulline accumulates in leaves of wild watermelon plants under drought (Kawasaki et al., 2000). Organisms other than plants also accumulate compatible solutes; for example, glycerol in yeast (Morales, et al. 1990) and phytoplanktons (Ben-Amotz and Avron, 1979), ectoine in *Halomonas* (Nakayama et al., 2000), trimethylamine *N*-oxide and urea in shallow-sea animals (Yancey et al., 2002), and di-*myo*-inositol-1,1'-phosphate and related compounds in thermophilic and hyperthermophilic bacteria and Archea (Santos and da Costa, 2002).

3.1. Functions of Compatible Solutes

The mechanisms by which compatible solutes protect cellular components from stresses are still obscure in many cases. However, increasing evidence suggests that an accumulation of compatible solutes in plants causes resistant to various stresses such as drought, high temperature and high salinity (Chen and Murata, 2002). Compatible solutes contribute to stress tolerance by acting as osmoregulators, since their high solubility in water acts as a substitute for water molecules released from leaves. In some cases, compatible solutes also act as active oxygen scavengers or thermostabilizers (Akashi et al., 2001; Kaushik and Bhat, 2003).

High concentrations of compatible solutes can increase cellular osmotic pressure (Delauney and Verma, 1993). Moreover, their high hydrophilicity helps maintain the turgor pressure and water content of cells and protect against water loss from leaves under drought. Compatible solutes, because of their extremely hydrophilic property, might also replace water molecules around nucleic acids, proteins and membranes during water shortages (Hoeskstra et al., 2001). Cell-water deficits cause an increase in the concentration of ions that destabilize macromolecules. Compatible solutes might prevent interaction between these ions and cellular components by replacing the water molecules around these components, thereby, protecting against destabilization during drought.

There is evidence showing that compatible solutes stabilize enzymes. For example, RuBisCO activity is suppressed by high concentrations of NaCl, but glycine betaine and proline can protect this enzyme against inhibition (Solomon et al., 1994; Nomura et al., 1998). Glycine betaine has also been shown to stabilize the PSII supercomplex in the presence of high concentrations of NaCl (Sakamoto and Murata, 2002). Plant cells might also use glycine betaine as a low-molecular weight chaperon as in *Escherichia coli* (Bourot et al., 2000). Trehalose exerts its function at lower concen-

trations than other compatible solutes. The resurrection plant *Myrothamnus flabellifolius* accumulates trehalose to increase the thermostability of its proteins (Kaushik and Bhat, 2003; Drennan et al., 1993), while resistance to various stresses is conferred in plant cells at low concentrations (Garg et al., 2002). Drought stress causes cellular membrane damage and leakage of ions from plant cells. Fructans, another compatible solute, have the ability to stabilize phosphatidylcholine liposomes during freeze-drying (Steponkus, 1984; Hincha et al., 2000).

Leaves close their stomata to avoid evaporation of water during drought, and consequently, the in-flow of CO_2 into the leaves stops. As a result, the sun's energy cannot be used for CO_2 fixation and instead is used for formation of active oxygen molecules in the chloroplasts. Superoxide and hydrogen peroxide are decomposed by enzymes specific to these active oxygen species. However, no enzyme has been shown to decompose hydroxyl radicals, the most dangerous of all active oxygen species. Some compatible solutes function as scavengers of hydroxyl radicals (Akashi et al., 2001; Shen et al., 1997). For example, it has been reported that levels of free radicals are decreased in tobacco plants transformed to accumulate more proline (Hong et al., 2000). The reactivity of citrulline and mannitol to hydroxyl radicals is much higher that that of proline; citrulline can promptly decompose all hydroxyl radical molecules at the formation site (Table 2) (Akashi et al., 2001).

Table 2 *. Second-order rate constants for reactions between hydroxyl radicals and various compounds*

Compound	*Rate constant ($M^{-1}s^{-1}$)*	*Concentration in vivo (mM)*	*Half-life of hydroxyl radicals generated in vivo (ns)*
Citrulline	3.9×10^{9}	200-300	0.59-0.89
Mannitol	2.1×10^{9}	100-320	1.0-3.3
Proline	5.4×10^{8}	120-428	3.0-11
Glycine betaine	8.2×10^{7}	320-1,000	8.5-26
Ascorbic acid	7.3×10^{9}	25-50	1.9-3.8
Glutathione	8.6×10^{9}	1-4.5	18-80

3.2. Biosynthesis of Compatible Solutes

The accumulation of metabolites can be accomplished by either promoted synthesis or repressed degradation, or both. The substrates of compatible solutes are often metabolites included in primary metabolic pathways with the flux of metabolites for synthesis of compatible solutes being highly controlled (Nuccio et al., 1999).

Proline is synthesized from glutamate through glutamate semialdehyde and Δ1-pyrroline-5-carboxylate (P5C) through P5C synthetase (P5CS) and P5C reductase (P5CR), respectively, the genes for which are up-regulated strongly under drought stress (Hare et al., 1999). The reaction catalyzed by P5CS is thought to be the rate-limiting step in the proline synthetic pathway, since an Arabidopsis mutant over-expressing this gene accumulated a high level of proline (Kishor et al., 1995), and the antisense sequence caused the synthesis of much fewer amino acid compared with the wild-type (Nanjo et al., 1999). The degradation of proline is catalyzed in the mitochondria by sequential reactions catalyzed by proline dehydrogenase (PDH) then P5C dehydrogenase (P5CDH), both of which are induced by proline accumulation in the cells (Deuschle et al., 2001). Thus, in plants, all genes for synthesis and degradation of proline are up-regulated where proline is accumulated. The accumulation of proline is tightly controlled and only achieved when the rate of synthesis prevails over that of degradation, probably because too much proline is toxic to cells.

Glycine betaine accumulates in various plants such as spinach, sugar beet and barley under drought, high salinity and cold stresses, and is synthesized from choline. Choline monooxygenase (CMO) converts choline into betaine aldehyde (BA), which is then metabolized to glycine betaine by BA dehydrogenase (BADH). The genes of these enzymes have been shown to be up-regulated by salt and drought stresses through an ABA signal transduction system (Ishitani et al., 1995; Rathinasabapathi et al., 1997). However, in *Arabidopsis* and tobacco plants, over-expression of these genes prevents synthesis and accumulation of glycine betaine to the level found in plants that naturally accumulate it (Nuccio et al., 1999; Huang et al., 2000; Holmstron et al., 2000). The reason has been ascribed to a shortage of the precursor for glycine betaine synthesis, namely, choline. Choline is synthesized from phosphoserine through phosphoethanolamine and phosphocholine. Phosphoserine is decarboxylated into phosphoethanolamine, which is then *N*-methylated three times by S-adenosyl-L-methionine:phosphoethanolamine *N*-methyltransferase (PEAMT) (Nuccio et al., 1999). The resultant phosphocholine is then hydrolyzed into choline. Expression of the PEAMT gene in spinach has also shown to be up-regulated by salt treatment (Nuccio et al., 2000). Moreover in tobacco, introduction of the PEAMT gene together with the genes for CMO and BADH increases the accumulation of glycine betaine 30-fold compared with the introduction of the latter two genes only (McNeil et al., 2001). Thus, an increase in the supply of choline together with up-regulation of CMO and BADH is thought to be important in the massive accumulation of glycine betaine in plants.

On the contrary, transgenic Arabidopsis and tobacco plants over-expressing the gene for *Arthrobacter* choline oxidase (CO) accumulate considerable amounts of glycine betaine and show tolerance to drought, high salinity and low and high temperatures (Sakamoto and Murata, 2002). The discrepancy between these experiments might be related to differences in the enzymatic properties of CMO and CO. For example, the K_m and V_{max} of spinach CMO and Arthrobacter CO are 0.1 mM and 24 nmole/mg protein/

min, respectively (Brouquisse et al., 1989; Burnet et al., 1995) and 1 mM and 12 μmol/mg protein/min, respectively (Ikuta et al., 1977). Moreover, the specificity (V_{max}/K_m) of the enzymatic reaction of *Arthrobacter* CO is 50 times higher than that of spinach CMO, suggesting that *Arthrobacter* CO is superior to spinach CMO with respect to glycine betaine production if the expressed protein levels of both genes are the same. Celery synthesizes both mannitol and sucrose as translocation sugars in source leaves. Mannitol is synthesized from fructose 6-phosphate, an intermediate of gluconeogenesis for sucrose synthesis in the cytosol, and thereby pathways of mannitol and sucrose syntheses compete for carbons from photosynthesis (Stoop et al., 1996). The accumulation of mannitol is accomplished partly by sucrose-induced suppression of the mannitol-catabolizing enzyme NAD-dependent mannitol dehydrogenase (MTD) (Stoop et al., 1996), the transcript level of which is also down-regulated by sucrose (Williamson et al., 1995; Prata et al., 1997; Zamski et al., 2001). Under non-stressful growth conditions, celery plants convert half their fixed CO_2 into mannitol, while the other half is used to produce sucrose; it is possible they preferentially use sugars to support central metabolism. Such sugar repression during mannitol degradation would allow large amounts of mannitol to be stored as a reserve carboxyhydrate. On the other hand, when plants experience stress, sucrose synthesis is accelerated and MTD activity is inhibited. In addition to this direct effect of sucrose, transcript levels of MDH are also reduced by ABA. Reducing equivalents not utilized under stress are transferred to the cytosol via the triose phosphate shuttle to promote reduction of mannose-6-phosphate to mannitol-1-phosphate, which is then converted to mannitol (Gao and Loescher, 2000).

3.3. Transgenic Plants

An increasing number of reports have documented successful creation of compatible solute-forming transgenic plants. These trials are important in furthering our understanding of the functions of compatible solutes and elucidation of their accumulation mechanisms. Model plants such as Arabidopsis and tobacco as well as crop plants such as rice and potatoes have been common recipients of the genes necessary for synthesis of compatible solutes. Resistance against various kinds of stresses such as drought, low and high temperatures, and high salt concentrations have been achieved through such experiments (Chen and Murata, 2002; Hare et al., 1998; Nuccio et al., 1999).

Stress-tolerant transgenic plants have been created by over-expressing genes of enzymes absent or rate-limited in the metabolic pathway. These genes are obtained from organisms that naturally accumulate the compatible solute in question, and are sometimes engineered to lose feedback regulation for massive accumulation (Hare et al., 1998; Nuccio et al., 1999). However, not all transgenic plants accumulate sufficient amounts of compatible solutes nor attain the positive trait, particularly in the field (Chen and Murata, 2002; Serraj and Sinclair, 2002). To overcome these hurdles, we need to know more about the regulatory mechanism of compatible solute synthesis. Infor-

mation on the intracellular sites of synthesis, mechanisms of accumulation and degradation of compatible solutes, the enzymatic properties of the enzyme of interest, their intracellular and intercellular transport mechanisms and the mechanism of synthesis of the building blocks of compatible solutes are important for the successful creation of stress-tolerant plants.

Physiological evaluation of transgenic plants manipulated to fortify tolerance to environmental physical stresses is also important. Generally, tolerance of transgenic plants to physical stresses is evaluated in a growth chamber or green house to minimize the influence of unrelated environmental factors. Numerous reports have demonstrated successful creation of stress-tolerant plants under such artificial conditions; however, we have yet to evaluate many of these results in the field.

4. SIGNAL TRANSDUCTION AND GENE EXPRESSION DURING WATER STRESS

4.1. Drought Sensing and Long-Distance Signalling

Plant roots have machinery that enables them to sense the dryness of soil and direct their tissues in the direction of moisture (hydrotropism) (Eapen et al., 2005). However, how root cells sense the moisture status of soil remains unclear. SLN1 and SHO1 have been identified as osmosensors that stimulate the synthesis of compatible solute glycerol through activation of HOG1, a MAP kinase, in yeast (Wurgler-Murphy and Saito, 1997). *Arabidopsis* ATHK1 is a member of the AHK histidine kinase family and complements sensibility to osmotic stress in yeast *sln1/sho1* double null mutants (Urao et al., 1999). However, whether ATHK1 functions as an osmosensor in plants awaits further experiments.

Water deficit signals released in the roots during water stress are delivered to the leaves through more than one signaling route; the major signal is abscisic acid (ABA). After sensing dryness, root tissues synthesize ABA through strong expression of the gene for 9-*cis*-epoxycarotenoid dioxygenase, the key enzyme in ABA synthesis (Qin and Zeevaart, 1999). ABA is transferred to the leaves through vascular tissues, decreasing stomatal conductance (Trejo et al., 1995) and modulating expression of various genes involved in adaptation to drying environments (Bray, 2002). Although ABA is involved in the altered expression of many genes, there are many examples of ABA-insensitive gene expression. The signaling molecule delivered from the roots to leaves during ABA-insensitive gene expression is, however, unknown.

4.2. Signal Responses in Guard Cells

Various biochemical events are induced in the guard cells in response to ABA; for example, an outflow of potassium and anionic ions and decrease in sucrose and malate

concentrations, and consequently, a reduction in stomatal aperture are observed (Figure 2) (Schroeder et al., 2001). Guard cells integrate external and internal environmental information through ABA-dependent and independent pathways to modulate stomatal aperture (Fan et al., 2004). These external and internal stimuli include light (quality and quantity), CO_2 level, air moisture, and leaf-air vapor pressure difference (Mott and Parthurst, 1991; Assmann et al., 2000).

Biotinated ABA induces stomatal closure by binding to the outer surface of guard cell plasma membranes (Yamazaki et al., 2003), strongly suggesting that the receptor of ABA is located on the surface of the plasma membranes. A 42-kDa protein has been identified as an ABA-binding protein from membrane fractions of the leaf epidermis of *Vicia fava* (Zhang et al., 2002), but whether this protein functions as a receptor of ABA under drought stress remains unknown.

Calcium ions act as a second messenger in intracellular signal transduction during ABA signaling (Schroeder et al., 2001). In-flow of calcium ions into the cytosol from the vacuole and extracellular space increases the cytosolic concentration of calcium ions in ABA-treated guard cells. The level of calcium ions oscillates at intervals of several minutes. This increase in calcium concentration is not observed in the ABA-insensitive mutants *abi1* and *abi2* (Allen et al., 1999). Calcium ions suppress inward potassium channels and activate inward anion channels; thereby playing a central role in stomatal closure (Blatt, 2000).

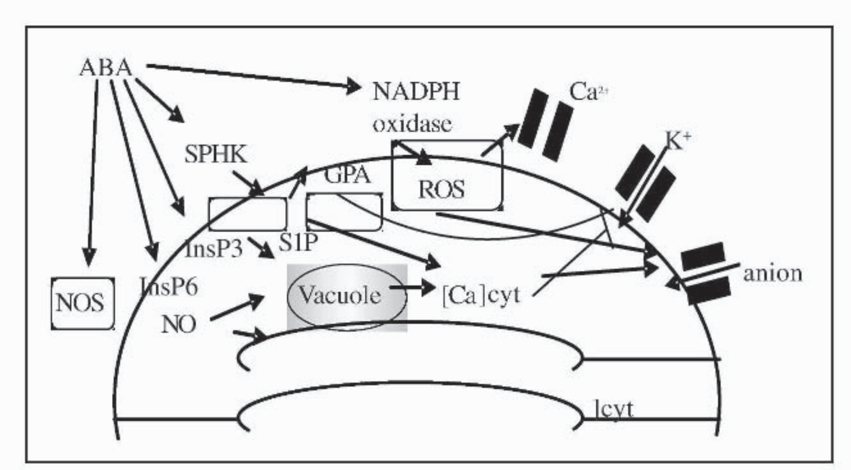

Figure 2*. The ABA signaling pathway in guard cells. Ion channels involved in stomatal closure are shown by a pair of black boxes, and protein factors involved in signal transduction are depicted in rounded squares. Abbreviations: ABA, abscisic acid; NOS, nitric oxidase synthase; NO, nitric oxide; InsP6, myo-inositol hexakisphosphate; InsP3, inositol 1,4,5-trisphosphate; SPHK, sphingosine kinase; S1P, sphingosine-1-phosphate; GPA, heterotrimetric G protein* α *subunit;* $[Ca^{2+}]cyt$*, cytosolic* Ca^{2+}

Abscisic acid increases the production of active oxygen species in the guard cells (Pei et al., 2000; Zhang et al., 2001). The involvement of NADPH oxidase in the production of active oxygen species has been demonstrated in the *atrbohD/F* Arabidopsis mutant in which the genes for putative subunits of NADPH oxidase have been disrupted (Kwak et al., 2003). The active oxygen species formed activate the calcium ion channel to increase the cytosolic concentration of calcium ions.

Another second messenger, sphingosine-1-phosphate (S1P), is formed from sphingosines, long-chain amino alcohols, by sphingosine kinase. ABA also increases activity of this enzyme in the guard cells (Coursol et al., 2003). S1P increases the intracellular concentration of calcium ions thereby contributing to stomatal closure (Ng et al., 2001). The *gpa* mutant of *Arabidopsis* in which the gene for the α-subunit of a trimeric small G protein has been disrupted, fails to inactivate the inward potassium channel and close its stomata even with administration of ABA or S1P (Wang et al., 2001). These findings support the model in which ABA signals are transferred to the ion channel through S1P and then the G protein. There is also evidence that ABA increases levels of other second messengers such as inositol 1,4,5-triphosphate (InsP3) and *myo*-inositol hexakisphosphate (InsP6). InsP3 and InsP6 also promote the release of calcium ions from the vacuole to cytosol thereby contributing to stomatal closure (Lemtiri-Chlieh et al., 2003).

It has also been proposed that nitric oxide (NO) functions as a second messenger in the ABA signaling pathway in guard cells. No stomatal closure occurs in the presence of ABA in Arabidopsis mutants lacking the NO synthase gene (Guo et al., 2003). The involvement of NO in stomatal closure has also been supported in other experiments where various reagents that stimulate NO production or inhibit NO metabolism have been applied (Garcia-Mata and Lamattina, 2002; Neill et al., 2002). It has been proposed that NO induces the efflux of calcium ions from the vacuole to cytosol during stomatal closure (Garcia-Mata et al., 2003).

4.3. Regulation of Gene Expression under Drought Stress

In response to drought stress, expression of a large number of genes is up-regulated (Figure 3) (Bray, 1997). Determination of drought-responsive genes in model plants such as Arabidopsis and rice was made possible after development of DNA micro-array technology (Seki et al., 2001, 2002; Kawasaki et al., 2001; Krebs et al., 2002; Leonhardt et al., 2004). Since some of these genes are also up-regulated by salt and low temperature stresses, signaling cascades for these stresses are thought to overlap.

Genes up-regulated by drought are categorized into two groups (Bray, 1997; Yamaguchi-Shinozaki and Shinozaki, 2005). One includes genes encoding proteins whose catalytic activities are responsible for protecting the cells and organs against stress, while the other includes genes encoding proteins necessary for signal transduction and regulation of gene expression. The proteins directly responsible for defending cells against stress participate in various physiological and biochemical events. They

include water and ion channels, enzymes necessary for the formation of compatible solutes, molecular chaperons, late embryogenesis abundant (LEA) proteins, proteins involved in protein degradation, and reactive oxygen-scavenging enzymes.

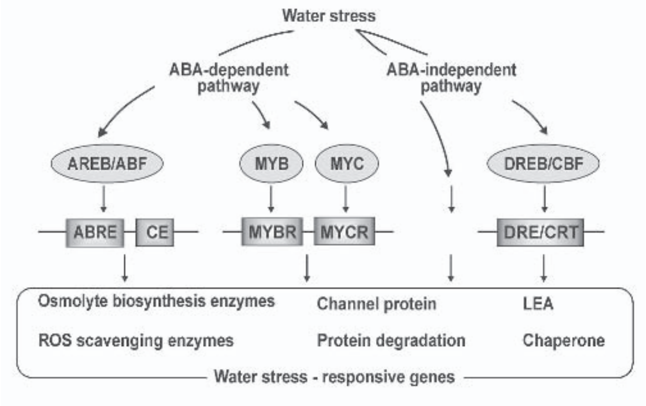

Figure 3. *Transcriptional factors and cis-acting elements involved in water stress-responsive gene expression. Transcriptional factors and cis-acting elements are shown in oval and square boxes, respectively. Abbreviations: ABA, abscisic acid; AREB, ABRE-binding proteins; ABRE, ABA-responsive element; CE, coupling element; MYBR, MYB recognition site; MYC, MYC recognition site; DREB, DRE-binding protein; CBF, C-repeat-binding factor; DRE/CRT, dehydration-responsive element/C-repeat; LEA, late embryogenesis abundant protein*

Expression of many genes for transcription factors is up-regulated under drought stress conditions. These transcriptional factors include the dehydration-responsible element (DRE)/C-repeat (CRT)-binding (DREB/CBF) protein family, ethylene-responsible element binding factor (ERF), zinc-finger family, WRKY family, basic helix-loop-helix (bHLH) family, basic-domain leucine zipper (bZIP) family, NAC family, and homeodomain transcription family. Drought stress also induces expression of proteins functioning in phosphorylation signaling cascades such as MAP kinase, calcium-dependent kinase, receptor kinase and histidine kinase, and in phospholipid metabolism (Zhu, 2002).

Drought-responsive genes can be divided into two groups, ABA-dependent and ABA-independent genes, according to their dependency on ABA for induction (Yamaguchi-Shinozaki and Shinozaki, 2005). The ABA-responsive element (ABRE) is a major *cis*-sequence in the promoter of many ABA-responsive genes. The *cis*-element was first identified in the *Em* gene of wheat and has since been found in various plants such as maize, oat, rice, tobacco and *Arabidopsis* where it functions as an ABRE. Expression of ABA-responsive genes is activated through binding of AREB/ABF, a bZIP-type transcription factor, to the ABRE sequence (Uno et al., 2000; Choi et al., 2000).

Both MYB- and MYC- recognition sites are present in the promoter region of the ABA-inducible gene RD22 of Arabidopsis. These sites are recognized by AtMYB2 and RD22BP1 (AtMYC2), respectively, and cooperatively activate RD22 gene expression (Abe et al., 1997). Since RD22 gene activation requires *de novo* protein synthesis, it has been suggested that MYB and MYC function at a later stage in the ABA-dependent drought response.

DRE/CRT is a *cis*-element involved in ABA-independent expression of drought-inducible genes. It was characterized with the promoter region of the RD29A gene in Arabidopsis (Yamaguchi-Shinozaki and Shinozaki, 1994). The *trans*-factors for the *cis*-element are CBF/DREB1 and DREB2 (Stockinger et al., 1997; Liu et al., 1998), which are expressed transiently soon after sensing drought and up-regulate the target genes involved in drought tolerance. These target genes have also been identified using DNA micro-arrays. Interestingly, rice homologues of these factors, OsDREB1 and OsDREB2, have also been shown to be involved in molecular responses to drought (Doubuzet et al., 2003).

Overlap between ABA-dependent and -independent signaling pathways has been suggested from detailed analysis of the promoter region in drought-responsive genes. For example, the promoter region of the *Arabidopsis* RD29 gene contains both DRE and ABRE *cis*-elements, and transactivation experiments with *Arabidopsis* leaf protoplasts suggest that both DREBs and ABREs cumulatively activate RD29 gene expression (Narusaka et al., 2003).

4.4. Molecular Responses to Drought in Drought-Tolerant Plants

Various wild plants inhabit harsh environments and have strong tolerance to a number of stresses. Accumulating evidence suggests that the molecular mechanisms of tolerance in these plants are quite different from those in model and domesticated crop plants.

Retama raetam is a C_3-type legume that grows wild in arid areas along the Mediterranean coast. Lower leaves of this plant are shaded from strong light and active in photosynthesis, while upper leaves suffer the stress of strong light. Proteins, such as RuBisCO, ascorbate peroxidase, and D1 of PSII, virtually disappear in these upper leaves during the dry summer season, and thus, become photosynthetically dormant (Mittler et al., 2001). However, mRNAs for these proteins persist in association with polysomes in the cells and are translated to original protein levels within 6 to 24 hours after supply of water. This drought-tolerance mechanism allows this plant to efficiently maximize its water utilization under water-deficit conditions.

There is a significant difference in the genes up-regulated during drought stress in drought-tolerant and domesticated or model plants. *Mesembryanthemum crystallinum* shows up-regulation of a gene for the enzyme, *myo*-inositol 1-phosphate synthase, involved in the initial metabolic step of pinitol synthesis (Ishitani et al., 1996); however, the homologous gene in *Arabidopsis* is not induced during drought. A

similar phenomenon has been observed in many other wild plants such as *Citrullus lanatus* (Akashi et al., 2004), *Lycopersicon pennellii* (Mittova et al., 2002), *Tortula ruralis* (Oliver et al., 2004), and *Xerophyta viscose* (Garwe et al., 2003).

During desiccation, the resurrection plant *Craterostigma plantagineum* changes its main photosynthetic product from sugar 2-octulose to sucrose, increasing the sucrose content to 40% of the dry weight of the plant body. Rehydration induces normal photosynthesis within 24 hours (Ingram and Bartels, 1996). The CDT-1 gene cloned from *C. plantagineum* by activation tagging was shown to be essential for dehydration tolerance in this species, and the level of its transcript was shown to increase in response to ABA (Furini et al., 1997). CDT-1 encodes a regulatory RNA or short peptide involved in activation of the ABA signal transduction pathway. No homologous gene has been found in the genome of *Arabidopsis*, suggesting a unique signal transduction system for adaptation to severe dehydration in this plant.

Research with drought-tolerant plants has revealed how plants have adopted a variety of signaling pathways that allow them to tolerate naturally dry environments. Information from these studies is expected to indicate genes that would be useful in future molecular breeding of crop plants.

5. CONCLUDING REMARKS

This chapter dealt with the basic responses of model plants to drought stress and the systems adopted by wild plants to environmental drought. However, the mechanisms for sensing environmental stimuli and transducing signals between organs and cells require further elucidation even in model plants.

Based on their relationship with water, plants can be divided into three categories: hydrophytes, mesophytes and xerophytes. Mesophytes survive in moist environments, and include most experimental model plants. Xerophytes can further be divided into two groups depending on their drought tolerance strategies. One group includes plants able to tolerate and resurrect following desiccation into photosynthetically active states in a short period of time, while the other is the desiccation-avoiding group. Plants in the latter group possess an improved water-absorbing system or water-storing organ, efficient water conduction within the plant body or a restricted transpiration system, or all or a combination of these features. Moreover, plants in this group are thought to have unique systems that allow stress tolerance, since they are able to defend their photosynthetic apparatus and other fragile cellular components from composite stresses in the harsh environments in which they are found. These plants are often inconvenient but promising experimental materials, since they are truly able to survive drought.

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CHAPTER 3

SALT STRESS

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1. INTRODUCTION

Saline water occupies 71% of the Earth area. It is thought that even a quarter of the whole pedosphere is affected by salts (Glenn and O'Leary, 1985), amounting to 950 x 10^6 ha (Flowers and Yeo, 1995), while 23 % of the 1.5 x 10^9 ha cultivated land is considered as saline (Rhoades and Loveday, 1990). Furthermore, about a half of all the existing irrigation systems of the world (3 x 10^8 ha) are under the influence of secondary salinization, alkalization and waterlogging, and about 10 x 10^6 ha of irrigated land are abandoned each year because of the unfavorable effects of secondary salinization and alkalization (Szabolcs, 1987). Such unfavorable soils of low fertility are generally unsuitable for agricultural production, causing unacceptable yield reduction, and in some cases, being far from any reasonable utilization. Because of the increased need for food production and increasing distribution of soils affected by salinity, research on plant responses to salinity has rapidly expanded in recent decades.

Studies of plant tolerance to salt stress cover many aspects of the influences of salinity on plant behavior, including alterations at the morphological, physiological and molecular levels. Recently, investigations are focusing more on: biotechnology, transgenic plants, improvement of breeding and screening methodologies and modification of the genetic structure of existing crops aiming at enhanced adaptation to salinity conditions.

The alterations in physiological traits caused by salt stress have been frequently reviewed during the past decades (Waisel, 1972; Flowers et al., 1977; Greenway and Munns, 1980; Munns et al., 1983; Ungar; 1991, Munns, 2002). However, the progress of research methodologies and techniques has created a platform for better understanding of molecular aspects (Yeo, 1998; Hasegawa et al., 2000; Tester and Davenport,

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2003) and genetic information related to this problem (Zhu, 2000, Yokoi et al., 2002*a;* Xiong and Zhu, 2002). The number of publications appearing on the effects of salinity on plants has continued to grow over the years, and now exceed 300 *per annum* (Flowers and Yeo, 1995).

Although salinity and sodicity are common phenomena for arid and semiarid regions of the world, salt-affected soils have been recorded in practically all the climatic regions, and in a wide range of altitudes. The term "salt-affected" refers to soils that are saline or sodic, and these cover nearly 10% of the total land surface (Pessarakli and Szabolcs, 1994). According to FAO Land and Plant Nutrition Management Service the total world area under saline and soils is 397 x 10^6 and 434 x 10^6 ha, respectively, and of the almost 1500 x 10^6 ha of dryland agriculture about 2% are salt-affected. The regional distribution of salt-affected soils was evaluated by FAO Land and Plant Nutrition Management Service (http://www.fao.org/ag/agl/agll/ spush/topic2.htm) (Table 1).

Table 1. *Regional distribution of salt-affected soils (in million hectares)*

Regions	*Total area*	*Salt-affected soils*	
	Mha	*Mha*	*%*
Africa	1899	73	3.4
Asia and Australia	3107	444	14.3
Europe	2011	80	3.9
Latin America	2039	112	5.5
Near East	1802	106	5.9
North America	1924	20	1.0
Total	12781	831	6.5
Secondary salinization in the world's irrigated lands		45.4	20

Water evaporates in a pure state leaving salts and other substances behind (Carter, 1975). Removal of water due to evapotranspiration leads to increased concentration of salts in soils. Salinity occurs through both natural and human-induced processes resulting in the accumulation of dissolved salts in the soil water and excess of sodium ions in the rhizosphere. Sodicity is a secondary consequence of salinity, typical for clay soils, where exchangeable sodium is bound to the negative charges of clay.

Salt-affected soils can be characterized as soils formed under the influence of different salts in their solid or liquid phases, which further affect the chemical, physical and biological features of the soil and, finally, its fertility (Pessarakli and Szabolcs, 1994). The fact that salt-affected soils are widespread throughout the world and that the problem of secondary salinization has been even more expressed due to various human

activities (mainly irrigation), as well as the strong negative effects of salinity on agriculture, saline and alkali soils have been broadly investigated from various aspects, including their genesis, physical and chemical properties, fertility, management and utilization (Kamphorst and Bolt, 1976; Bresler et al., 1982; Szabolcs, 1989; Pessarakli and Szabolcs, 1994).

Natural or primary salinity results from the accumulation of soluble salts in soils or groundwater over long geological periods, mainly by weathering of parent minerals, releasing salts of various types, such as chlorides, sulfates, carbonates and bicarbonates of sodium, magnesium and calcium (Richards, 1954). Besides the naturally-formed saline and sodic soils, the occurrence of so-called secondary salt-affected soils is becoming ever more visible, due to application of different agricultural practices, mainly irrigation.

However, there are human influences, other than irrigation, that lead to adverse effects of secondary salinization, such as: overgrazing, deforestation in semi humid and semiarid areas, contamination with chemicals and accumulation of airborne or waterborne salts (Pessarakli and Szabolcs, 1994).

2. HALOPHYTES VERSUS GLYCOPHYTES

Based on general tolerance to salt stress, all plants can be roughly divided into two major groups: a) halophytes, that can withstand even 20% of salts in the soil and, in most cases, successfully grow in conditions with 2-6% of salts (Strogonov, 1964), and b) non-halophytes or glycophytes, plants that exhibit various degrees of damage and limited growth in the presence of sodium salts, usually higher than 0.01%. However, there are great differences in the level of salt stress tolerance within both the halophytes (Waisel, 1972; Flowers et al., 1977; Munns et al., 1983; Ungar, 1991) and non-halophytes (Greenway and Moons, 1980), which include sensitive, moderately tolerant and very tolerant species. Although halophytes represent only 2% of the terrestrial plant species, they are present in about half the higher plant families and exhibit a great diversity of plant forms (Glenn et al., 1999).

Many groups of plants are considered as sensitive (e.g. conifers, ferns, Orchidaceae, Araceae, Rosaceae, Ericaceae and molds), while particular families comprise tolerant genera and species, such as Potamogetonaceae, Plumbaginaceae, Zygophyllaceae, Frankeniaceae, Tamaricaceae, Rhizophoraceae, etc. (Waisel, 1972). It is interesting that the widespread Chenopods may be designated as halophytes "per excellence" (Flowers and Yeo, 1988), as half of the total genera successfully grows in conditions of salinity (*Atriplex*, *Suaeda*, *Salsola*, *Camphorosma*, *Salicornia*, etc.).

Regarding the origin of halophytes there are two hypotheses, such species either migrated form coastal habitats to the inland (Chapman, 1960), or spread from inland steppes (e.g. *Statice* ssp.) to the coastal saline regions (van der Pijl, 1969). In any case, the wide distribution of halophytes throughout the world indicates their polyphyletic origin (Flowers et al., 1977), while distinct changes in the genome during evo-

lution have enabled more active regulation of uptake, transport, accumulation and utilization of ions in order to maintain the water balance and ion homeostasis.

There is no distinct classification of halophytes. Different authors (e.g. Chapman, 1960; Strogonov, 1973; Waisel, 1972) have tried to distinguish some specific groups of salt-tolerant species, such as, oligo-, meso- and euhalophytes, obligate and facultative halophytes, succulent, salt-excreting, non-succulent halophytes, etc. Each categorization of plant tolerance to salt stress is based upon particular criteria, but global differences in responses to salinity are mainly dependent on adaptive strategies gathered during evolution and natural selection. Salt tolerance relies upon mechanisms at all-organizational levels of the plant. Although numerous similar cellular mechanisms are present in both halophytes and non-halophytes for growth in saline conditions (Binzel et al., 1989), the halophytic species seem to be more efficient in utilization of different salt tolerance mechanisms. Halophytes rely predominately on osmotic adjustment through utilization of ions as osmolytes with active sequestration into the vacuoles (e.g. Flowers et al., 1977; Yeo, 1998; Glenn et al., 1999). For example, in the halophytes *Salsola soda* L., *Suaeda maritima* (L.) Dum., and *Camphorosma annua* Pall., contribution of ions (Na^+, Cl^-, K^+ and SO_4^{2-}) to the osmotic potential ranged from 68.7% to 80.38% according to survey carried out in the conditions of their natural habitat (Dajic et al., 1998).

3. MAIN ADAPTATIONS OF PLANTS TO SALT STRESS

Adaptive strategies of plants exposed to salinity are based upon the utilization of one or more of the following major mechanisms (Levitt, 1972; Munns et al., 1983; Fitter and Hay, 1989; Niu et al., 1995):

(1) Phenological avoidance (related to plants which complete their cycle of growth and development in the most favorable period of the vegetation season);
(2) Salt avoidance through salt exclusion, which can be achieved by low root permeability for certain ions, especially sodium;
(3) Salt avoidance through secretion, which is dependent on the presence of special salt glands and bladders;
(4) Dilution of high salt concentration in plant tissues by succulence and growth, which is, among other things, related to the flexibility of cell walls;
(5) Active accumulation and compartmentation of salts into the vacuoles;
(6) Biochemical tolerance through adaptations of cell organelles and macromolecular systems to excess of salt;
(7) Nutritive tolerance (the capacity for metabolic utilization of potassium and calcium ions in order to mitigate the adverse effects of sodium ions)

In summary, mechanisms of salt tolerance are of two main types: those minimizing the entry of salt into the plant (or at least their accumulation in photosynthetic tissues) and those minimizing the concentration of salt in the cytoplasm (Munns, 2002). This corresponds with two major adaptive strategies of plants to tolerate high environmental salinity: 1) stress avoidance, related to different physical, physiological and/or metabolic barriers with which the adverse effects of stress are ameliorated, and 2) stress tolerance, the linkage of adaptive mechanisms which enable successful survival despite the influence of stress internally. It is clear that the regulation of Na^+ uptake and transport across the plasma membranes and tonoplast will be a key factor determining the plant cell response to salinity stress.

4. LIMITING SALT ACCUMULATION

4.1. Salt Exclusion

Salt exclusion is a very efficient but complex way of preventing massive ion uptake in the root zone, enabling a lower uptake and accumulation of salts in the upper parts of the plant, especially in the transpiring organs. Salt exclusion is based upon lower root permeability for ions even in the presence of high external salinity.

It is often found that many glycophytes, when exhibiting enhanced tolerance to salinity stress, have a greater ability for sodium exclusion from the shoot and for maintaining high levels of K^+ (e.g. Zhu, 2001; Flowers and Hajibagheri, 2001). Salt sensitive plants, such as beans and maize are the most prominent Na^+ excluders (Jacoby, 1994; Bayuelo-Jimenez et al., 2003). The growth reduction in maize was caused by rapid salt accumulation in older leaves, where a more salt-tolerant cultivar exhibited a higher ability for exclusion of toxic ions (Fortmeier and Schubert, 1995). Additionally, in moderately tolerant crops, such as bread wheat, salt tolerance is associated with low rates of sodium transport to the shoots and high K^+/Na^+ discrimination (Gorham, 1990), which was also shown for the tetraploid *Triticum turgidum*, subspecies *durum* (Munns et al., 2000; Munns and James, 2003). Among graminaceous crops, more or less proficient in salt exclusion, rice may be an exception, because of large rates of sodium influx into the roots under salinity stress, which was ascribed to leakage past the endodermis (Yeo et al., 1999). The level of Na^+ entry into the root through leakage via the apoplast in rice was about ten times greater than this bypass flow in wheat (Garcia et al., 1997). Although it is considered that this mechanism of salt exclusion is mainly characteristic for the species less tolerant of salt stress, halophytic plants exclude salts as well (Munns, 2002). An example is mangrove *Avicennia marina* with a degree of salt exclusion of 98% (Ball, 1988). It was demonstrated that at 200 mM external Na^+, about 97% of all Na^+ presented to the root surface must be excluded, whether in a glycophyte or halophyte, thus showing the necessity of restraining of Na^+ uptake and accumulation in the shoots (Munns et al., 1999).

The strategy of salt exclusion relies on the selective release of Na^+ into the xylem and its resorption from the xylem stream. Net accumulation of sodium ions in the plant is dependent on the balance between passive influx and active efflux. Salt exclusion operates at the cellular and whole plant level (Munns et al., 1983) and is to a great extent related to regulation of K/Na selectivity (Jeschke and Hartung, 2000). According to Munns et al. (2002), the ability of plants to regulate the uptake and transport of salts is dependent on the following mechanisms: a) selectivity of uptake by root cells, b) preferential loading of K^+ rather than Na^+ into the xylem by the cells of the stele, c) removal of salts from the xylem in the upper parts of roots, the stem and leaf sheaths, based upon exchange of K^+ for Na^+, and d) loading of the phloem.

Passive movement of ions into roots and shoots is the consequence of the transpirational stream (Flowers and Yeo, 1992). However, at the endodermis, radial movement of solutes must be via the symplast, and the extent to which the symplastic pathway of ion transport regulates reduced delivery of ions into the xylem is still not known (Clarkson, 1991). The endoderm zone represents the main barrier of passive flow of ions towards the shoot. Thus, in some halophytic species the level of suberization of the endoderm cell walls reaches 50-100%, compared with non-halophytes with 27-40% (Osmond et al., 1980).

Besides the role of the cortex and endodermis, transfer root cells are also important in the process of control of sodium ions movement (Greenway and Munns, 1980). It has been suggested that in order to minimize Na^+ delivery to the shoot in the apoplast of the xylem, cells in the outer half of the root need to suppress the influx from and/or increase efflux to the soil solution, in contrast to the cells of the inner half of the root, which should maximize influx from and/or minimize delivery of sodium ions to the xylem (Tester and Davenport, 2003).

Radial transport of sodium from the soil solution into the xylem vessels might be genetically controlled, according to results obtained in *Arabidopsis* sas1 mutants (for sodium over-accumulation in the shoot) compared with the wild-type plants (Nublat et al., 2001). Apart from the regulation of xylem loading, controlled by Na^+/H^+ antiporters, retrieval of ions from the xylem may also operate, probably due to the Na^+-permeable channel of xylem parenchyma cells (Wegner and Raschke, 1994).

The salt tolerance in species that exclude salts is achieved by changes between sodium and calcium ions, rather than changes in osmotic potential, since adsorption of calcium ions on membranes of root cells leads to reduced penetration of monovalent cations (Munns et al., 1983). This was demonstrated for wheat where inhibition of non-directional Na^+ influx occurred following the addition of external Ca^{2+} (Reid and Smith, 2000). Involvement of both Ca^{2+} sensitive and Ca^{2+} insensitive pathways (regulated mainly by non-selective cation channels) in the control of Na^+ entry into the root has been proposed (Tester and Davenport, 2003).

The search for mechanisms of active sodium extrusion in root cells has to continue, as, in contrary to algae, advanced protocols for the isolation of rhizodermal protoplasts and PM vesicles are needed (Gimmler, 2000). An important goal of salt

tolerance research is to determine those transporters which function in the control of Na^+ entry, to find a way to limit Na^+ influx and thus to improve salinity tolerance (Zhu, 2001).

4.2. Significance of K/Na Selectivity

Uptake and distribution of sodium ions within the root is to a large extent connected with the effects of potassium, since Na^+ efflux in root cortex cells is stimulated by K^+ influx, which is related to the K/Na root selectivity (Jeschke, 1972). The presence of potassium (and calcium) ions has been shown to decrease Na^+ influx into plant cells (e.g. Lazof and Bernstein, 1999).

In wheat, salt tolerance is related to the enhanced K/Na selectivity (Gorham, 1990) and it has recently been reported that the genus *Triticum* expresses a range of genetic variation related to K/Na discrimination. The trait is highly heritable (Munns et al., 2002). In contrast to dicotyledonous plants, in monocots the maintenance of a lower Na/K ratio in shoots is of greater significance, because of their lower capacity for sodium storage and higher requirement for K^+ and compatible organic solutes (Glenn et al., 1999).

Time-course measurements of root ion content have indicated the possibility of a sodium-excluding mechanism in roots of *Kosteletzkya virginica* through low Na^+/K^+ ratios brought about not only by exclusion of Na^+, but also by a strong K^+ affinity which was ascribed to a change of the sterol/phospholipid ratio in salinized roots (Blits and Gallagher, 1990). A wide range of K^+/Na^+ selectivity and sodium exclusion were demonstrated for halophytic and non-halophytic Chenopods (Reimann, 1992), with Na^+ accumulation being most pronounced in the shoots.

To confer salt stress tolerance, in many species, the achievement of a high K^+: Na^+ ratio is more important than simply maintaining low concentrations of sodium ions (Maathuis and Amtmann, 1999). A highly significant correlation between K^+ and Na^+ in leaves and salinity-induced yield reduction was reported for a number of rice genotypes irrigated with moderately saline water (Asch et al., 2000). The significance of a high K/Na ratio for maintenance of yield components and fiber characteristic has also been evaluated in salt-tolerant cotton lines (Ashraf and Ahmad, 2000). The maintenance of higher K^+ and Ca^{2+} to Na^+ ratios, especially in young growing and recently expanded tissues, appeared to be important characteristics of salt tolerance in barley cultivars (Wei et al., 2003). In a study with four barley cultivars exposed to a range of salinity concentrations (Figure 1) it was noticed that the highest differences in Na^+: K^+ ratio, were expressed at 200 mol m^{-3} NaCl (Dajic, unpublished data). The Na^+ : K^+ ratio seems to be valuable criterion for screening of salt tolerance within salt-sensitive species, such as maize. It was found that among four maize cultivars differing in drought tolerance, and tested for their responses to salt stress (Dajic, unpublished data), the Polj 17, previously characterized for high capacity of ABA accumulation under drought stress and related tolerance to water deficit (Pekic and Quarrie, 1988) has maintained the lowest values of Na/K under salinity conditions (Figure 2).

This is also true for the highly tolerant wild relative of wheat, *Lophopyrum elongatum*, whose chromosome arms 1ES, 7ES and 7EL were shown to play a role in enhanced K^+/Na^+ selectivity in wheat, according to studies using the amphiploid with wheat cultivar Chinese Spring (Deal et al., 1999).

4.3. Ion Retranslocation, Allocation and Leaching

Retranslocation of ions via the phloem is a potentially important mechanism of preventing salt accumulation in fully expanded leaves, while at the same time it represents a threat to younger leaves, which are phloem sinks. It seems that cells of salt-sensitive plants lack Na^+/H^+ transporters at the tonoplast, and therefore, cannot efficiently sequester any excess of ions into the vacuoles (Jacoby, 1994). Instead, they

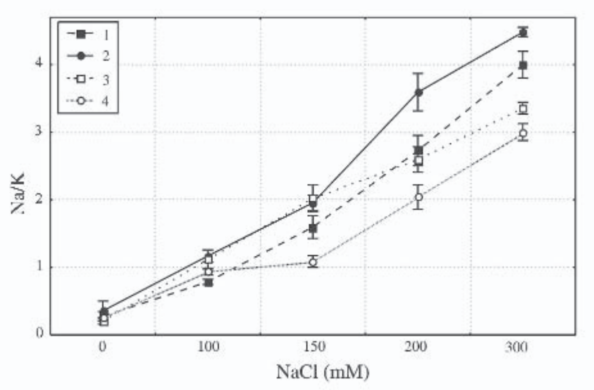

***Figure 1.** The Na: K ratio in leaf 4 of barley cultivars exposed to salinity (1- cultivar Jelen, 2- NS 310, 3- NS 430, and 4- Viktor)*

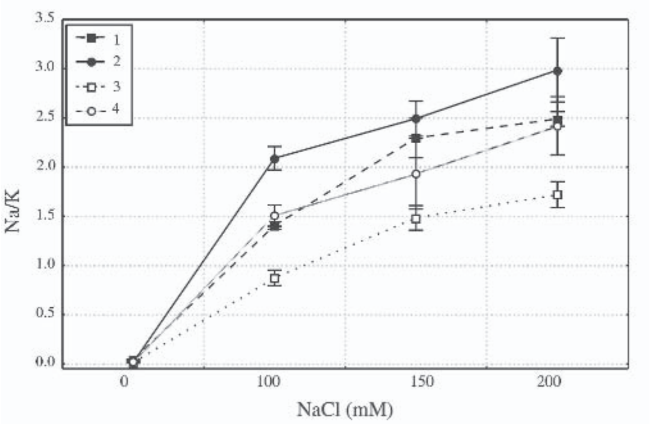

***Figure 2.** The Na: K ratio in leaf 3 of maize cultivars exposed to salinity (1- cultivar B73, 2- DTP79, 3- Polj17, 4- F2)*

need to rely on sodium recirculation to exclude sodium salts from the shoot. Export of ions from shoot to root via the phloem has been demonstrated in beans (Greenway and Munns, 1980), *Trifolium alexandrinum* (Winter, 1982), maize (Lohaus et al., 2000), barley (Munns et al., 1986), cotton (Gouia et al., 1994) white lupin (Munns et al., 1988) and *Lycopersicon pennellii* (Perez-Alfocea et al., 2000). Phloem export may remove at least 25% of the total Na^+ intake by the leaf, especially when growth decreases and demands for ions diminish (Munns et al., 1983).

One possibility for Na^+ retranslocation is the coupling to inverse H^+-gradients created by H^+-ATPases, where Na^+/H^+ antiporters utilize these gradients by exchanging external H^+ for internal Na^+, i.e., secondary energized Na^+ export (Gimmler, 2000). These properties suggest that Na^+, which enters the symplast, probably by passive diffusion from external solution of high concentration, is relatively effectively pumped from the symplast, either into the external solution or into the stele (Osmond et al., 1980). Thus, the efflux of sodium ions out of root cells might be associated mainly with the activity of Na^+/H^+ antiporters (Blumwald et al., 2000).

Besides salt exclusion in the roots, low NaCl levels in leaves can also be achieved by salt retention in the lower plant parts and also through abscission of old leaves once they accumulate large quantities of salts (Flowers and Yeo, 1992; Munns, 1993). In beans, ions accumulate in the root or in the basal part of the shoot, from where they are returned to the root system and excreted back into the medium (Jacoby, 1979). The mechanism of intra-plant allocation is also characteristic for many halophytes, which, due to the limited transpiration, can keep excess of salts within their roots and lower parts of the shoot, thus preventing ion accumulation in the photosynthetic tissues (e.g. Waisel, 1972; Dajic, 1996).

The significance of ion leaching from the leaf apoplast, through the cuticle, by rain, fog or dew (Tukey, 1970) is still unclear (Pennewiss et al., 1997). Control of the ion uptake is certainly achieved through restriction of the transpiration. Plants transpire 30-70 times more water than they use for cell growth, which means that solutes will be in the same degree concentrated by the roots of non-excluder species (Munns, 2002). It was postulated that partial stomatal closure observed in *Aster tripolium* under high salinity is induced by the presence of sodium ions in the apoplast surrounding the guard cells (Kerstiens et al., 2002), causing a reduction in rates of transpiration and increase of water use efficiency.

4.4. Salt Excretion

Salt excretion is also a very efficient way of preventing excessive concentrations of salts building up in photosynthetic tissues. This mechanism is typical for species that have developed special features, mostly localized at the leaf epidermis, known as salt glands and salt hairs (bladders). One of the most obvious signs of salt excretion is the salt crust on leaves and shoots of those species with salt glands or salt hairs (Popp, 1995).

These structures are common for many halophytic genera such as *Cressa* (Convolvulaceae), *Frankenia* (Frankeniaceae), *Spartina*, *Chloris*, *Aeluropus* (Poaceae), *Atriplex* (Chenopodiaceae), *Statice*, *Limonium*, *Plumbago*, *Armeria* (Plumbaginaceae), *Glaux* (Primulaceae), *Tamarix*, *Reamuria* (Tamaricaceae), as well as, some mangrove species, e.g. *Avicennia*, *Aegialitis*, *Aegiceras* and *Acanthus* (Waisel, 1972; Crawford, 1989; Popp, 1995).

Glandular structures involved in salt excretion vary in structure, position, physiological mechanism and related ecological significance. The simplest are the two-celled (*Spartina*, *Aeluropus*) and three-celled types (*Chloris gayana*), while more the complex are structures composed of 5-9 cells (*Avicennia*), 8 cells (*Tamarix*) and, especially, 16 cells in some representatives of the family Plumbaginaceae (Waisel, 1972; Crawford, 1989). The following basic types of salt excreting structures might be recognized: two-celled glands of the grasses, multicellular glands of various dicotyledonous halophytes and bladder hairs in some Chenopods (Thomson et al., 1988). They all contain one or more subtending cells that are in apoplastic and symplastic connection with both the adjacent mesophyll and the distal, secreting gland cell (Jacoby, 1994).

Glandular structures are usually spread over the whole surface area of the shoot, though they are most abundant on the leaves. Differentiation of the salt- excreting structures is generally completed before differentiation of the entire leaf, indicating the importance of salt glands in survival during the early developmental stages of the plant (Waisel, 1972). There is still a dilemma about the salt glands, whether they functions to excrete, secrete, or recrete (Freitas and Breckle, 1992; Marcum and Murdoch, 1992). There are a lot of similarities in the metabolic principles of ion transport and functioning of the proton pumps in salt-excreting structures and cells of other tissues. Features that control free ion transport, analogous to the Casparian strips of the endodermis, have also been found in salt glands (Breckle et al., 1990). The activity of salt glands may be induced by an increase of external salinity, which has been shown by a 30% increase in the activity of H^+-ATPase isolated from salt glands (Hill and Hill, 1973). Dschida et al. (1992) identified an energy dependent process in salt excretion, achieved by plasma membrane H^+-ATPase in *Avicennia germinans*. The chemical composition of the secretion present in salt glands (inorganic ions and organic compounds, such as sugars, amino acids and amines) indicates the possibility of leakage from the protoplasm caused by a disturbed structure of membrane systems of the cell during the process of excretion (Ziegler and Lüttge, 1967).

Besides the active pumping of ions into the salt glands from the palisade and water parenchyma cells, it seems that simultaneous vesicular transport of ions leading to excretion into the extracellular space and direct salt secretion into the subcuticular area are operating, as shown in *Avicennia marina* (Ish-Shalom Gordon and Dubinsky, 1990). Although the apoplastic pathway in solute transport to the gland cell prevails, symplastic transport, documented through the presence of Cl^- in the plasmodesmata of gland cells of *Limonium* (Ziegler and Lüttge, 1967) also operates. Additionally, the significance of vesicle-mediated solute transport in salt excretion has been suggested

(Echeverria, 2000). The efficacy of salt glands has been established by the presence of low concentrations of salts in leaves and a high K/Na ratio of species possessing such structures, even in conditions of rapidly increasing external salinity (Bradley and Morris, 1991).

It seems that in salt-secreting plants the mechanism of salt exclusion is operating as well, since it was demonstrated that in mangrove plants *Aegiceras corniculatum* and *Avicennia marina* Na^+ excretion does not keep pace with Na^+ uptake over a range of salinities, reducing plant growth at high external salinity (Ball, 1988).

Salt bladders, probably best studied in several species of the genus *Atriplex* (Chenopodiaceae), are also of particular importance in the regulation of salt tolerance. They are composed of two cells: the small, basal and the upper, bladder cell (Osmond et al., 1980). The latter dies, once it has accumulated a sufficient amount of salt in its vacuole, leaving a salt crust at the leaf surface (Figure 3).

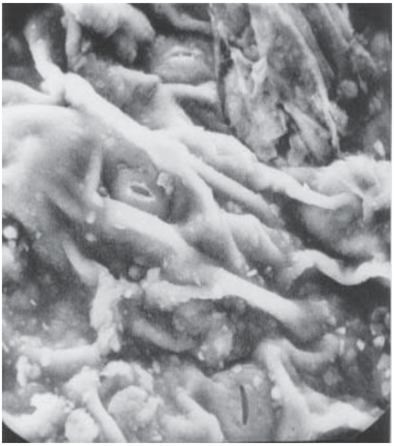

Figure 3. *Leaf surface of the salt-excreting halophyte Atriplex tatarica L. var. diffusa Ten. (x 1000, Dajic, 1996)*

Many *Atriplex* species are characterized by the presence of salt bladders at the surface of young leaves only (Breckle et al., 1990), and these, in same cases, may contain almost the whole leaf sodium (Jeschke and Stelter, 1983). It is considered that the salt bladders of *Atriplex* represent a key adaptation to salt tolerance for their natural habitats (Freitas and Breckle, 1992).

Altogether, the prevention of excessive salt accumulation within the plant body is achieved through the following modes, such as: a) control of the salt uptake at the root level and regulation of Na^+ delivery to the shoot by loading of the xylem and retrieval of ions from the xylem before reaching the shoot), b) K/Na selectivity, c) recirculation of salts via the phloem, d) allocation of salts within particular parts of the plant, e) ion leakage and abscission of organs loaded with salts, f) control of transpiration, and g) secretion of ions by salt-excreting structures.

5. SALT ACCUMULATION

High accumulation of salts in the shoot has been well established for salt- tolerant species (e.g. Waisel, 1972; Flowers et al., 1977; Ungar, 1991). In halophytes of the plant community *Puccinellietum limosae* in Serbia, which is spread on highly salinized soil (annual values of EC_e ranged between 20.8 dS m^{-1} and 54.2 dS m^{-1}), the level of total salt accumulation in the shoot during the vegetative season (June-October), and the relations among accumulated ions (Table 2) were strictly species specific, depending on different adaptive strategies (Dajic, 1996), such as salt accumulation in the halophytes *Suaeda maritima* and *Salsola soda*, and salt exclusion in the halophytic grass *Puccinellia limosa*.

Table 2. *Average seasonal ion concentrations of the shoot (ìmol g^{-1} dry weight) in halophytes grown in natural habitat conditions*

Species	Na^+	K^+	Mg^{2+}	Cl^-
1	660.9 ± 317.88	438.5.1± 162.96	155.2 ± 30.32	478.9 ± 180.71
2	843.5 ± 375.38	467.3 ± 182.67	168.7 ± 32.20	615.5 ± 263.23
2	2686.9 ± 982.06	341.5 ± 86.87	391.7 ± 132.81	1877.7 ± 614.2
4	791.3 ± 234.78	365.6 ± 95.41	158.3 ± 49.31	555.5 ± 244.18
5	1345.7 ± 608.58	363.5 ± 142.98	87.5 ± 22.05	504.9 ± 188.21
6	1834.8 ± 664.11	375.2 ± 99.21	125.2 ± 56.3	901.4 ± 538.31
7	127.5 ± 64.06	157.3 ± 54.82	61.1 ± 17.33	177.5 ± 65.2

1- Atriplex tatarica, 2- *Atriplex litoralis*, 3- *Suaeda maritima*, 4- *Aster tripolium* var. *pannonicus*, 5- *Camphorosma annua*, 6- *Salsola soda*, 7- *Puccinellia limosa*
(after Dajic, 1996)

5.1. Vacuolar Compartmentation

The high content of salts in aboveground parts of the halophytic plants is feature associated with efficiency of such plants to deliver ions into the vacuoles. The ability of plants to transfer ions into the vacuole is dependent on the proportion of highly vacuolated cells and tissues, as well as the activity of transport systems located at the tonoplast, which prevent excessive concentration of ions in the cytoplasm. Sequestration of salts into the leaf and/or shoot vacuoles is typical attribute of dicotyledonous halophytes, coupled with other physiological adaptations, such as regulation of transpiration (and water regime in general) and performing of cell metabolism with low potassium concentrations (Flowers and Dalmond, 1992).

Therefore, the capacity of vacuolar compartmentation, which enables effective osmotic adjustment of plants grown in saline conditions, and liberation of cytosol free from the presence of toxic ions, is one of the key factors in salinity tolerance. Many halophytic species are characterized by their large vacuoles. The vacuoles occupy 77% of the mesophyll cells of *Suaeda maritima* (Hajibagheri et al., 1984) and are capable of accumulating salts to concentrations higher than 500 mM (Dracup and Greenway, 1985). In a study with *S. maritima* conducted on its natural habitat in Serbia, sodium concentration of the cell sap exceeded even 800 mM, while the total salt content contributed to the osmotic potential in degree of up to 91 % (Dajic et al., 1997*b*).

In the case of salt sequestration into the vacuole, potassium ions and organic solutes should be accumulated in the cytoplasm in order to achieve and maintain the osmotic and ionic balance between these two compartments (Flowers et al., 1977; Greenway and Munns, 1980; Munns, 2002).

5.2. Succulence

The feature of succulence is characteristic for many dicotyledonous halophytes (Crawford, 1989), and represents a very efficient way of mitigation of adverse osmotic and toxic effects of ions through dilution. In natural conditions, the content of salts in vegetative organs of halophytes increases with ageing, and might reach toxic levels. Therefore, halophytic species tend to lower such unfavorable salt concentrations by increasing of water content in their tissues and/or by enhanced growth. Morpho-anatomical alterations of succulent halophytes include increase of cell volume, especially of spongy and water parenchyma, increase of leaf thickness and decrease in number of stomata (Strogonov, 1973). The significance of phenotypic plasticity in adaptations to salt stress was documented in experiments with *Plantago coronopus*, where salt treatment lead to increased leaf thickness and leaf dry matter percentage (Smekens and Vantienderen, 2001). Shoots of halophyte *Salicornia bigelovii* were larger and more succulent when grown in highly saline conditions (Parks et al., 2002). Salt-induced succulence was also reported for two subspecies of *Salsola kali*: subsp. *kali* and subsp. *tragus*, where the latter, when grown in saline medium, exhibited about three times higher leaf thickness compared with the control plants (Rilke and Reimann, 1996).

The type of salinity plays a role in the occurrence of particular alterations of plant structure. For example, chloride salinity contributes to the succulent forms, while sulfate salinity leads to xeromorphism (Strogonov, 1964, 1973). Succulence is important also in regulation of temperature and water balance, especially in warm and dry periods of the year (Fitter and Hay, 1989), and this has been established for many halophytes of extremely dry saline habitats (Weiglin and Winter, 1991). The ability of halophytes to dilute salts both through succulence and growth is dependent on cell wall extensibility, enabling the increase of cell volume followed by further uptake of water in order to balance the excess of salts (Munns et al., 1983). Hence, in order to maintain growth under saline conditions, plants may either increase the amounts of

solutes in the cell and regulate turgor or adjust plasticity and threshold turgor (the latter are both cell wall characteristics). Succulence is associated with the ability of intracellular compartmentation, to provide a larger capacity (volume of vacuoles) for salt storage.

The effective capacity of halophytes to accumulate and utilize ions for osmotic adjustment to maintain turgor, might explain their enhanced growth and control of their water regime in saline conditions (e.g. Greenway and Munns, 1980; Dajic, 1996; Yeo, 1998; Glenn et al., 1999), although the osmotic benefits of storing ions are limited both by the available vacuolar space and metabolic costs of ion pumping into the vacuoles (Lerner, 1999). Many halophytes express growth stimulation under moderate salinities where concentrations of salts for optimum growth vary among species, but generally may be as high as 400 mM or more (Tester and Davenport, 2003). For example, the halophyte *Suaeda fruticosa* exhibits much greater fresh and dry weights under saline conditions (200 to 400 mM NaCl), compared with plants grown in non-saline media (Khan et al., 2000*a*). The salt-tolerant grass *Anneurolepidium chinense* successfully survived upon exposure to 500 mM NaCl and had developed stolons for daughter plants (Ochiai and Matoh, 2001). The presence of NaCl induced increases in fresh and dry weight of 100% and 30%, respectively, in the evergreen perennial halophyte *Salvadora persica* (Maggio et al., 2000). It was shown that halophytes, such as: *Batis maritima*, *Distichlis spicata*, *Juncus roemerianus*, *Paspalum vaginatum*, *Salicornia bigelovii* and *Spartina alterniflora* successfully develop at up to 20g L^{-1} of mean salinity of the soil solution with minimum reduction in the growth potential (El-Haddad and Noaman, 2001). Nevertheless, Yeo and Flowers (1980) suggested that growth stimulation of halophytes by the presence of salt is somewhat arbitrary and should be considered separately from tolerance towards extreme salinities, which is a case of survival.

6. GROWTH AND DEVELOPMENT OF PLANTS UNDER SALINITY CONDITIONS

There is general agreement that whole plant growth responses to salinity are multigenic and that a better knowledge of the underlying physiology is required in order to understand why some species and varieties are more salt-resistant than others (Neumann, 1997). This is a complex task since plant growth responses to salinity can vary with: 1) the duration and degree of stress encountered (mild, moderate, severe), 2) experimental system used, i.e. the plant organ, variety or species, and 3) the plant developmental stage. Some species are more tolerant at the seedling stage, while the other exhibit higher tolerance during vegetative growth, flowering or fruiting (e.g. Subbarao and Johansen, 1994). Moreover, the growth inhibitory effects of salinity can also be affected by variation of calcium or potassium ions in the saline root medium (Neumann, 1997).

The mechanisms by which salinity inhibits shoot growth may be grouped into the following categories (Lazof and Bernstein, 1999): 1) disturbed photosynthesis, 2) decline in turgor of expanding tissues and insufficient osmoregulation, 3) root sensing and down-regulation of shoot growth via a long distance signal, and 4) disturbance in mineral supply to the shoot.

Reduced plant growth under saline conditions is at least partly due to high concentrations of salt building up in the apoplast of growing tissues. Nevertheless, high ion concentrations in the apoplast may lead to cell and tissue dehydration, as per Oertli hypothesis (Oertli, 1968). High accumulation of ions in the apoplast has been reported for salt-sensitive plants, such as rice (Flowers et al., 1991). Under salt treatment, apoplastic ion concentrations of more tolerant spinach were much lower than in less tolerant pea (Speer and Kaiser, 1991). However, there was no evidence for high accumulation of sodium in the leaf apoplast of maize and cotton exposed to salinity conditions (Mühling and Läuchli, 2002*a*), suggesting the existence of specific adaptive responses in different species.

Munns (1993) has suggested that plant growth under salinity is inhibited through two phases. Initially (phase 1), growth is affected because of cellular responses to the osmotic effects. In the subsequent phase (phase 2), growth is reduced due to the toxic effects of accumulated salts.

Time-dependent changes of growth and development of plants exposed to salinity stress have been reviewed (Munns, 2002). In the first few seconds or minutes, cells lose water and shrink, whereas over hours cells regain their volume, but the expansion rates are limited. Over days and weeks, reduced cell elongation and cell division result in slower leaf appearance and inhibition of shoot growth. Certainly, the ability to withstand salinity stress over a longer period of time would be dependent on complex mechanisms of stress tolerance, especially those, which prevent salt reaching toxic levels in photosynthetic tissues. Thus, the relative rates of appearance of new leaves and the death of old leaves might be crucial for plants to enter their reproductive period (Munns, 2002).

Plant growth is direct result of intensive division and expansion of meristematic cells. The primary response to salinization is associated with the rate of Na^+ transport to the shoot apical meristem and other processes in the plant might be affected before an increase in sodium concentrations within the growing tissue, particularly sensitive to salinity (Lazof and Bernstein, 1999). Leaf elongation rate was shown to decline rapidly under salinity conditions, with inhibition of cell extension exerted by changes in the yield threshold of the cell and not by turgor (Cramer, 1992). Leaf emergence rate has also been reported to be very sensitive to salinity even in salt-tolerant species, such as *Atriplex amnicola*, where the number of emerging leaves decreased continuously as salinity level increased (Aslam et al., 1986). Complex physiological changes such as cell wall extensibility and osmotic adjustment are involved in the early inhibition of growth in expanding plant tissues exposed to osmotic stress (Neumann, 1997).

Alterations in nutritional status under salinity conditions, on the basis of the concentrations of Na^+ and K^+ in growing tissues, disturbed calcium, and the status of other nutrients in young tissues, as well as, the comparative effects between young and mature tissues, have been reviewed by Lazof and Bernstein (1999).

General inhibition of shoot growth with continued root growth has been considered as a morphological adaptation to salt or water stress (Saab et al., 1990). An increase of root/shoot ratio during the vegetation season was observed in the halophyte *Suaeda maritima*, which might be a consequence of both ageing and increased soil salinity (Dajic et al., 1997*b*).

6.1. Growth of Crops under Salt Stress

There are great varietal differences in plant growth responses to salinity, depending on genotypic differences in rates of salt uptake, transport, accumulation and distribution within the plant. For example, the shoot dry weight of three oat cultivars progressively decreased under increasing concentrations of salinity (Dajic, unpublished data). The most evident differences in growth responses to salinity were observed at the treatment of 100 mM NaCl, whereas at higher salt concentrations a considerable reduction of shoot dry weight (about three to five times lower at 150 mM and 300 mM, respectively, than those of the control plants) occurred in all tested cultivars (Figure 4).

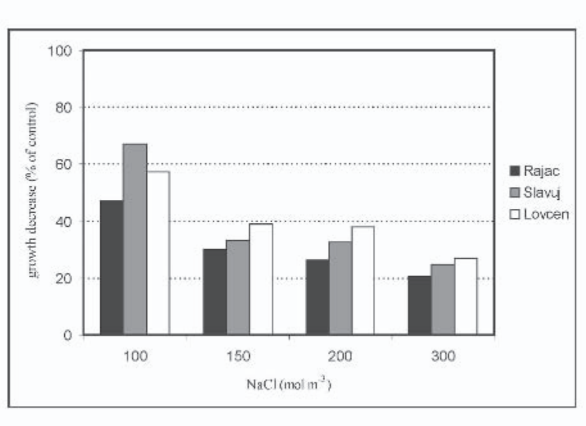

Figure 4. *Growth reduction of oat cultivars (cv. Rajac, Slavuj and Lovcen) caused by increasing salt concentrations*

The salt tolerance of crop species, including cereals, forage crops, fruit crops and vegetables (Table 3) is conventionally expressed (Maas and Hoffman, 1977) in terms of relative yield (Y_r, i.e. the percentage of the yield of the crop grown under saline conditions relative to that in non-saline conditions), threshold salinity value (a), that is, the maximum soil salinity that does not reduce yields below those produced under non-saline conditions, and slope (b), the relative reduction per unit salinity increase from threshold (soil salinity is expressed in terms of EC_e), as follows:

$$Y_r = 100 - b\,(Ec_e - a) \qquad (1)$$

Table 3. *Salt tolerance of crops (cereals, forage crops, vegetables and fruit crops)*

Common name	*Botanical name*	*Threshold* Ec_e (dSm^{-1})	*Slope* % per dSm^{-1}	*Rating*
Alfalfa	*Medicago sativa* L.	2.0	7.3	MS
Almond	*Prunus ducilis* L.	1.5	19.0	S
Apple	*Malus sylvestris* L.	-	-	S
Apricot	*Prunus armeniaca* L.	1.6	24.0	S
Asparagus	*Asparagus officinalis* L.	4.1	2.0	T
Barley	*Hordeum vulgare* L.	8.0	5.0	T
Beet, red	*Beta vulgaris* L.	4.0	9.0	MT
Bean	*Phaseolus vulgaris* L.	1.0	19.0	S
Bermuda grass	*Cynodon dactylon* (L.) Pers.	6.9	6.4	T
Blackberry	*Rubus* sp.	1.5	22.0	S
Broad bean	*Vicia faba* L.	1.6	9.6	MS
Cabbage	*Brassica oleracea* L.	1.8	9.7	MS
Canola	*Brassica napus* L.	-	-	T
Carrot	*Daucus carota* L.	1.0	14.0	S
Castorbean	*Ricinus communis* L.	-	-	MS
Celery	*Apium graveolens* L.	1.8	6.2	MS
Cherry	*Prunus avium* L.	-	-	S
Chickpea	*Cicer arietinum* L.	-	-	MS
Clover, alsike	*Trifolium hybridum* L.	1.5	12.0	MS
Clover, red	*Trifolium pratense* L.	1.5	12.0	MS
Clover, sweet	*Melilotus sp*. Mill.	-	-	MS
Clover, ladino	*Trifolium repens* L.	1.5	12.0	MS
Corn	*Zea mays* L.	1.7	12.0	MS
Cotton	*Gossypium hirsutum* L.	7.7	5.2	T
Cucumber	*Cucumis sativus* L.	2.5	13.0	MS
Currant	*Ribes sp*. L.	-	-	S
Eggplant	*Solanum melongena* L.	1.1	6.9	MS
Fig	*Ficus carica* L.	-	-	MT
Flax	*Linum usitatissimum* L.	1.7	12.0	MS
Garlic	*Allium sativum* L.	1.7	10.0	MS
Grape	*Vitis sp*. L.	1.5	9.6	MS
Kenaf	*Hibiscus cannabinus* L.	8.1	11.6	T
Lemon	*Citrus limon* L.	-	-	S
Lettuce	*Lactuca sativa* L.	1.3	13.0	MS

Table 3. *Continued...*

Muskmelon	*Cucumis melo* L.	1.0	8.4	MS
Oats	*Avena sativa* L.	-	-	T
Olive	*Olea europaea* L.	-	-	MT
Onion	*Allium cepa* L.	1.2	16.0	S
Orange	*Citrus sinensis* L.	1.7	16.0	S
Parsnip	*Pastinaca sativa* L.	-	-	S
Pea	*Pisum sativum* L.	3.4	10.6	MS
Peach	*Prunus persica* L.	1.7	21.0	S
Peanut	*Arachis hypogaea* L.	3.2	29.0	MS
Pear	*Pyrus communis* L.	-	-	S
Pepper	*Capsicum annuum* L.	1.5	14.0	MS
Plum	*Prunus domestica* L.	1.5	18.0	S
Pomegranate	*Punica granatum* L.	-	-	MT
Potato	*Solanum tuberosum* L.	1.7	12.0	MS
Pumpkin	*Cucurbita pepo* L.	-	-	MS
Radish	*Raphanus sativus* L.	1.2	13.0	MS
Raspberry	*Rubus idaeus* L.	-	-	S
Rice	*Oryza sativa* L.	-	-	S
Rye	*Secale cereale* L.	11.4	10.8	T
Rye grass, Italian	*Lolium multiflorum* Lam.	-	-	MT
Rye grass, perennial	*Lolium perenne* L.	5.6	7.6	MT
Sorghum	*Sorghum bicolor* L.	6.8	16.0	MT
Soybean	*Glycine max* (L.) Merrill	5.0	20.0	MT
Spinach	*Spinacia oleracea* L.	2.0	7.6	MS
Strawberry	*Fragaria vesca* L.	1.0	33.0	S
Sugar beet	*Beta vulgaris* L.	7.0	5.9	T
Sugar cane	*Saccharum officinarum* L.	1.7	5.9	MS
Sunflower	*Helianthus annuus* L.	-	-	MT
Timothy	*Phleum pratense* L.	-	-	MS
Tomato	*Lycopersicon esculentum* L.	2.5	9.9	MS
Triticale	x *Triticosecale* Wittmack	6.1	2.5	T
Turnip	*Brassica rapa* L.	0.9	9.0	MS
Wheat	*Triticum eastivum* L.	6.0	7.1	MT

(adapted after Maas, 1986, 1990 and Francois and Maas, 1994; T- tolerant, MT- moderately tolerant, MS-moderately sensitive, S- sensitive)

6.2. Nitrogen Fixation and Salt Stress

Legumes represent a very significant group of crops in agriculture, and therefore their responses to salt stress are described in several reports (e.g. Katerji et al., 2001, Lachaal et al., 2002). Tolerant varieties and accessions within the legumes have been revealed, such as for soybean (Essa, 2002) and among both cultivated and wild *Phaseolus* species (Bayuelo-Jimenez et al., 2002). Breeding and genetic engineering programs of legumes must be directed to optimise their nitrogen fixation and growth in saline conditions.

Establishment of symbiosis is highly sensitive to salt stress, whereas fully developed nodules that had been formed under salt stress can continue to fix nitrogen (Singleton and Bohlool, 1984). Host tolerance was a major factor for nodulation and nitrogen fixation in genotypes of faba bean (Cordovilla et al., 1995). Rhizobia can survive under much higher salinity than its host legume (Nair et al., 1993). Bacterial ability to adapt to salt stress is important for the bacteroid nitrogen-fixing function inside the legume nodule. Among several *Rhizobium* species tested for salt stress tolerance, *R. meliloti* has expressed a much higher ability to survive in the saline medium, comparing to *R. leguminosarum* and, especially *R. japonicum* (Bernard et al., 1986).

Salt stress tolerance in rhizobia is at least partially associated with osmoregulation achieved by accumulation of compatible solutes (Imhoff, 1986). Inn conditions of salt stress, pea plants treated with boron and calcium exhibited enhanced cell and tissue invasion by *Rhizobium leguminosarum* and increased nodule number (El-Hamdaoui et al., 2003). Furthermore, enzymes involved in ammonium assimilation in root nodules exhibited a significant sensitivity to salt stress and should not be considered as reliable criteria for selection of salt tolerance in faba bean (Cordovilla et al., 1995) and pea (Cordovilla et al., 1999). Activity of nitrogenase, nodule number and dry matter accumulation in soybean (Abdalla et al., 1998) and alfalfa (Serraj and Drevon, 1998) were affected under salt stress.

Eight genes of *Rhizobium tropici* were involved in salt tolerance and establishment of symbiosis with beans. These were classified into three groups: a) two genes responsible for regulation of gene expression and regulation of nitrogen metabolism, b) genes related to synthesis or maturation of proteins, and c) genes associated with potassium uptake and polysaccharide biosynthesis (Nogales et al., 2002).

7. WATER REGIME AND PHOTOSYNTHESIS UNDER SALT STRESS

The effects of drought and salt stress on plants are tightly related, since the first responses of plant cells under these stress conditions are induced by osmotic shock. Thus, upon exposure to osmotic stress, plants exhibit many common adaptive reactions at the molecular, cellular and whole-plant level (Greenway and Munns, 1980; Yeo, 1998; Bohnert et al., 1995; Zhu et al., 1997). These include morphological and anatomical alterations (life cycle, xeromorphic features, increased root/shoot ratio), and physi-

ological traits associated with maintaining water relations and photosynthesis (e.g. different pathways of carboxylation, such as C_4, intermediate C_3-CAM and CAM) (Dajic et al., 1997*a*). Additionally, various metabolic changes, such as the maintenance of ion and molecular homeostasis (e.g. synthesis of compatible solutes necessary for osmotic adjustment), detoxification of harmful elements and growth recovery, which depends mainly on various signaling molecules, occur under exposure to salt/drought stress (Xiong and Zhu, 2002).

Increasing salinity in the growth medium decreases content of chlorophyll and the net photosynthetic rate, which is expressed more conspicuously in salt-sensitive plants, such as alfalfa (Khavarinejad and Chaparzadeh, 1998) and canola (Qasim et al., 2003). Under salinity treatment, two wheat cultivars expressed two phases of photosynthetic inhibition: in the first phase, photosynthetic reduction was gradual, whereas in the second phase it was rapid and accompanied by a decline of the energy conversion efficiency in photosystem II, strongly related to adverse effects of salinity (Muranaka et al., 2002). Reduction of net CO_2 assimilation with salinity in tomato and sunflower was related to decrease in stomatal conductance and stomatal density (Romeroaranda et al., 2001; Rivelli et al., 2002*b*). The decrease was due to reduced CO_2 assimilation associated with a decline in stomatal conductance, water use efficiency and Rubisco activity, as well as slower electron transport of photosystem II under severe salt stress.

In many halophytic species regulation of the water regime is associated with the type of CO_2 fixation. Certain halophytes, originating from the tropics and subtropics, utilize the CAM (Crassulacean Acid Metabolism) pathway of carboxylation. Water availability is the major selective factor for evolution of the CAM pathway in plants, where nocturnal CO_2 fixation saves loss of water by transpiration and increases water-use efficiency (Larcher, 1995). Induction of the CAM pathway in the common ice plant (*Mesembryanthemum crystallinum*) under stress conditions is dependent on its biochemical machinery, which enables an increase in PEP-carboxylase and other CAM enzyme activities (Michalowski et al., 1989, Thomas et al., 1992), as well as enzymes involved in synthesis of compatible solutes, particularly pinitol (Vernon and Bohnert, 1992). The change from C_3-photosynthesis to CAM in *M. crystallinum* is elicited by salt stress and drought (Winter and Lüttge, 1979), and the kinetics of CAM induction depends on the strength of the stress and the developmental stage of the plant (Cushman et al., 1990*a*). Moreover, the stress-induced switch from C_3 to CAM may be linked with the ABA-induced activity of vacuolar ATPase in adult plants, while vacuolar Na^+ compartmentation is regulated through ABA-independent pathways in *M. crystallinum* (Barkla et al., 1999). The perennial cactus *Cereus validus*, having constitutive CAM, exhibits adaptations at the whole-plant level which differ from those of the annual CAM-inducible common ice plant, for example regulation of turgor and gas exchange, and metabolic adjustment at the cellular level and molecular level (Lüttge, 1993). Evaluation of signal transduction events involved in the induction of CAM in the common ice plant revealed that transcript abundance of *Ppc1*, a gene encoding the CAM-specific isoform of phosphoenol pyruvate carboxylase, rapidly increased during osmotic stress (Taybi and Cushman, 1999).

A significant number of halophytes are C_4 species, which are characterized by their higher requirements for sodium ions compared with C_3 species (Brownell and Crossland, 1972). In conditions of osmotic stress and high temperatures, C_4 plants have an advantage in comparison with C_3 plants, because of their ability to carry on photosynthesis when stomata are to a large extent closed, coupled with the absence of photorespiration in the mesophyll cells (Larcher, 1995). Photosynthetic responses to salinity in the halophytic tribe *Salsoleae* (family Chenopodiaceae) have been reviewed, with particular attention paid to relations between the C4 NAD-ME (malic enzyme) *Salsoloid* type of carboxylation and the chloroplast structure (Voznesenskaya et al., 1999).

Abscisic acid is well recognized as an important stress hormone. The concentration of ABA increases when water deficits occur, with its *de novo* synthesis beginning in the roots, in response to sensing an insufficient supply of water (Zhang et al., 1989). In halophytes, which grow in conditions of “physiological drought”, due to low water potential in the root medium, the lowest concentrations of ABA were found under salinity concentrations optimum for growth (Clipson et al., 1988). Such case was reported for the highly tolerant halophytic species *Suaeda maritima*, which exhibited the lowest seasonal range of ABA contents (from 649.4 ng g^{-1} to 835.6 ng g^{-1} dry weight) in comparison with several other species, where higher ABA concentrations were correlated with increased sodium content of the shoot (Dajic et al., 1997*a*).

In glycophytes, salinity leads to the accumulation of ABA (Asch et al., 1995), as in tomato (Chen and Plant, 1999; Yurekli et al., 2001) and wheat (Aldesuquy and Ibrahim, 2002). In bush bean plants exposed to 75 mM NaCl, inhibition of leaf expansion was mediated by ABA rather than by Na^+ or Cl^- toxicity, and the increase of ABA induced by a salt-pretreatment limited the accumulation of Na^+ and Cl^- in the leaves, resulting in adaptation to salinity stress (Montero et al., 1997). Besides the significant role of ABA, favorable effects of other hormones in plant responses to salinity, such as cytokinins (Kuiper and Steingrover, 1991) and gibberellins (Kaur et al., 1998; Ashraf et al., 2002) have been documented.

8. MOLECULAR BASIS OF SALT TOLERANCE

According to Hasegawa et al. (2000) determinants of salt stress tolerance include effector molecules that enable adaptive reactions and mechanisms of plants in saline environments and regulatory molecules that control these pathways. Effectors are proteins and metabolites involved in ion homeostasis (membrane proteins involved in regulation of ionic transport), osmotic adjustment and water regime regulation (osmolytes) and toxic radical scavenging (mainly enzymes), while regulatory molecules are cellular signal pathway components and transducers of long-distance response coordination (hormones, mediators, transcription factors and regulatory genes).

8.1. Biochemical Determinants of Salt Tolerance – Enzymes, Compatible Solutes and Protection Factors

The cytotoxicity of sodium lies in the high charge/mass ratio of the sodium ion (compared with potassium), causing disruption in water structure and a decrease in hydrophobic interactions and hydrostatic forces within proteins (Pollard and Wyn Jones, 1979). Additionally, Na^+ affects the activity of enzymes either by direct binding to inhibitory sites or by displacing K^+ from activation sites. It has been suggested that more than 50 enzymes are activated by K^+, and Na^+ can't be replaced in this function (Bhandal and Malik, 1988). Additionally, K^+ is needed for protein synthesis, as binding of tRNA to ribosomes requires K^+ (Blaha et al., 2000).

The effects of salts on enzymatic reactions are multiple and complex, although to a large extent, their influence is related to the change in cytosolic pH which strongly affects the activity of enzymes. It is generally accepted that enzymes exhibit slightly increased activity under low concentrations of ions, whereas they start to be inhibited in the presence of NaCl concentrations higher than 100mM (Munns, 2002). For instance, the activity of DNAse and RNAse in alfalfa and lentil seedlings was inhibited in the presence of 100 mM NaCl (Yupsanis et al., 2001).

Enzymes of halophytes are, in general, just as sensitive as enzymes of glycophytes (Greenway and Osmond, 1972; Flowers et al., 1977), but some salt tolerant plants exhibit *in vitro* tolerance of some enzymes to high concentrations of salts in (Flowers and Dalmond, 1992). However, the relevance of any assay under *in vivo* conditions is uncertain. Enzymes of cell wall compartment could be more salt-tolerant than cytoplasmic enzymes of higher plants (Thiyagarajah et al., 1996).

The salt tolerance of plants, irrespective of the sensitivity of enzymes and protein synthesis to high salt concentrations, is significantly related to the sequestration of salts into the vacuoles, which allows the normal activity of metabolic machinery in the cytoplasm. Salt-induced increases in the activity of enzymes involved in defense to oxidative stress are related to the reactive oxygen species scavenging pathway which takes place in the particular cell compartments, such as chloroplasts, peroxisomes, glyoxysomes and cytosol (Yeo, 1998; Rathinasabapathi; 2000, Xiong and Zhu, 2002), which, in difference to the vacuoles, do not accumulate the salts.

The cytosolic apparatus of both halophytes and glycophytes is very sensitive to osmotic and ionic effects of salts. Adverse effects of salts on the cell metabolism may be alleviated through synthesis and accumulation of compatible solutes and protection factors of macromolecules (mainly LEA proteins and chaperones). Accumulation of compatible solutes in response to salt stress is a metabolic adaptation, which primarily serves for osmotic adjustment and osmotic balance between vacuole and the cytosol. As found in a number of stress-tolerant species, there is a possibility of convergent evolution for this trait (Yancey et al., 1982; Rhodes and Hanson, 1993).

Compatible solutes are defined as organic osmolytes, which are compatible with the cell's metabolism, referring to protein/solute interactions and stabilization of

macromolecules, irrespective of species and nature of the stress (Yeo, 1998; Nuccio et al., 1999; Rathinasabapathi, 2000; Hasegawa et al., 2000; Huang et al., 2000). These Compatible solutes comprise a wide range of organic compounds, such as: simple sugars (fructose and glucose), sugar alcohols (glycerol and methylated inositols), complex sugars (trehalose, raffinose and fructans), polyols, quaternary ammonium compounds (proline, glycine betaine, â-alanine betaine, proline betaine) and tertiary sulfonium compounds (Rhodes and Hanson, 1993; Nuccio et al., 1999). As compatible solutes are hydrophilic, they can replace water at the surface of proteins, complex protein structures and membranes, which explains their action as osmoprotectants and as low-molecular-weight chaperones (Hasegawa et al., 2000).

Within this group of molecules, glycine betaine is a ubiquitous protein-stabilizing osmolyte occurring in all organisms (Rhodes and Hanson, 1993). Glycine betaine is an amphoteric compound, electrically neutral over a wide range of pH and extremely soluble in water, allowing it to interact with both hydrophilic and hydrophobic regions of macromolecules (Sakamoto and Murata, 2002). Glycine betaine a accumulated in many halophytic species, such as *Suaeda maritima* (Clipson et al., 1985), *Atriplex nummularia*, *Spergularia marina*, *Salicornia europea* (Stumpf, 1984) and *Salsola soda* (Manetas, 1990) in order to balance the osmotic potential difference between vacuole and the cytoplasm (Flowers et al., 1977). The accumulation of glycine betaine in halophytes (e.g. *Atriplex griffithii*) is induced by salt stress and increases with a raise of salinity (Khan et al., 1998; 2000b) in the halophyte. Glycine betaine was the major organic osmolyte of non-halophytes, such as wheat (Saneoka et al., 1999), red-beet (Subbarao et al., 2001), and sorghum (Yang et al., 2003). Despite its wide presence in many speeies, glycine betaine is absent in some crops, (rice) and tobacco (Yeo, 1998).

In higher plants the biosynthesis of glycine betaine is seeds as by two-step oxidation of choline (via the toxic intermediate betaine aldehyde) catalyzed by choline monooxygenase (CMO) and betaine aldehyde dehydrogenase (BADH), respectively (Sakamoto and Murata, 2002). The activity of these enzymes is localized to the chloroplast stroma, although some BADH activity was found in the cytoplasm (Weigel et al., 1986). Two kinds of BADH found in the mangrove halophyte *Avicennia marina* were characterized by high efficiency in the oxidation of betaine aldehyde (Hibino et al., 2001). Installing of genes involved in the synthesis of glycine betaine has had a certain success in improvement of salt tolerance in plants.

Polyols, such as glycerol, mannitol, sorbitol and sucrose, are osmoprotectants in algae and certain halophytic plants (Yancey et al., 1982). The biosynthesis and accumulation of specialized polyols (myo-inositol, D-ononitol and D-pinitol) in the cytosol of the stress-tolerant common ice plant (*Mesembryanthemum crystallinum*) was reported to increase with salinity (Nelson et al., 1999).

The sugar alcohol mannitol may serve as a compatible solute in salinity conditions. The role of mannitol in salt stress tolerance was evaluated in the non-mannitol producer *Arabidopsis* by installing the *M6PR* gene (for synthesis of mannose-6-phosphatase reductase) from celery, and in the presence of NaCl, mature transgenic plants

completed their normal development, including flowering and seed production even at salinity of 300 mM (Zhifang and Loescher, 2003). Enhanced salinity tolerance of egg-plant was achieved through installing of the bacterial *mt1D* gene encoding the mannitol phosphodehydrogenase, an enzyme involved in the mannitol synthesis (Prabhavathi et al., 2002).

Trehalose is a non-reducing disaccharide that functions as a compatible solute under abiotic stress in bacteria, fungi and invertebrates (Yeo, 1998). Until recently, trehalose had only been found in a few resurrection (desiccation- tolerant) plants, indicating the role of this molecule in adaptations to water stress due its ability to act as a water substitute on the surface of macromolecules (Öko- Institut, http://www.plantstress.com/Articles/up_general_files/GE_Tol.pdf). Trehalose accumulates in plants in a very low concentrations, and might be involved in the ROS scavenging and signaling cascade (Flowers, 2004). The overexpression of *Escherichia coli* trehalose biosynthetic genes (*otsA* and *otsB*) in transgenic rice resulted in sustained plant growth, lower photo-oxidative damage and a more favorable mineral balance under exposure to salinity (Garg et al., 2002).

Proline is a significant compatible solute in many halophytic species (Stewart and Lee, 1974), as well as in glycophytes, like *Medicago sativa* (Fougere et al., 1991) and *Sorghum bicolor* (McCree, 1986). Accumulation of proline in rice is a symptom of salt stress injury, and is due increase of the ornithine delta-aminotransferase (OAT) and its precursor glutamate (Lutts et al., 1999). Intermediates of proline biosynthesis and catabolism induced expression of several osmotically regulated genes in rice (Iyer and Caplan, 1998). The content of transcripts of two cDNA clones from alfalfa, encoding the first enzyme of proline biosynthesis pathway, *MsP5Cs-1* and *MsP5Cs-2*, increased in seedlings exposed to 90 mM NaCl (Ginzberg et al., 1999). Transgenic wheat plants producing proline due to the expression of genes for proline biosynthesis, transferred from *Vigna aconitifolia,* exhibited improved tolerance to salinity (Sawahel and Hassan, 2002).

Different classes of proteins of uncertain biochemical function (possibly macromolecule protection factors) are synthesized under conditions of salt stress, such as: osmotins, dehydrins, late embryogenesis abundant proteins (LEA) and polyamines, primarily putrescine and spermine (Tester and Davenport, 2003). Under salinity treatments, a balance between content of the free and bound polyamines in roots of barley seedlings might be relevant for salt tolerance (Zhao et al., 2003). There is no much data on the function of osmotins and dehydrins in conditions of salinity, but they may be involved in the maintenance of the protein structure (Campbell and Close, 1997). The late embryogenesis abundant-like proteins (LEA) accumulate in the vegetative tissues of all plant species in response to osmotic stress, caused by drought, salinity or cold (Xiong and Zhu, 2002). They probably contribute to the preservation of the structural integrity of the cell (Winicov, 1998), acting as chaperones to prevent denaturation of proteins (Xiong and Zhu, 2002). Most of the LEA-like proteins of all organisms are involved in adaptations to osmotic stress, and interestingly, express features of ribosomal proteins that interact with RNA (Garay-Arroyo et al., 2000).

8.2. Ion Homeostasis and Regulation of Sodium Transport

Ion homeostasis in conditions of salt stress is related to the activity and regulation of intrinsic membrane transport proteins, such as ATPases, carrier transporters and ion channels (Braun et al., 1986; Niu et al., 1993; Amtmann and Sanders, 1999; Krol and Trebacz, 2000; Blumwald et al., 2000; Tester and Davenport, 2003). There is no evidence for the existence of a Na^+-ATPase (postulated to be a primary Na^+ extrusion pump) in algae and higher plants, with the exceptions of the unicellular marine algae *Tetraselmis viridis* and *Heterosigma akashiwo* (Gimmler, 2000).

Sodium transport from the environment into the plant cells is a passive process, since the negative electrical potential differences at the plasma membrane and low concentration of sodium ions in the cytosol are the major forces for sodium uptake (Blumwald et al., 2000). Sodium uptake thus, depends on the electrochemical gradient of Na^+ and the presence of Na-permeable channels in the plasma membrane. Sodium ions may accumulate in the cytoplasm to 100 times the external concentration. Such accumulation in salt-tolerant plants is prevented by control of influx (channel gating) and/or active export from the cytoplasm to the vacuoles, and also outside from the plant (Jacoby, 1994). This means that Na^+ extrusion and vacuolar compartmentation are active processes. Active sodium transport in plant cells is performed by Na^+/H^+ transporters, which are ordinarily driven by an ATPase derived proton motive force.

8.2.1. Role of H^+-ATPases

Electrophoretic flux across the cell membranes and secondary active transport are facilitated by H^+ pumps, including H^+-ATPase of the plasma membrane and H^+-ATPase and H^+-pyrophosphatase of the tonoplast (Sze et al., 1999). The dominant ion pump at the plasma membrane of higher plants is a H^+- pumping ATPase, which provides the electrochemical potential difference for H^+ ions across the plasma membrane. Several genes are known to be involved in encoding H^+-ATPases (Michelet and Boutry, 1995). Proton-translocating activity was doubled in root cells of the halophyte *Atriplex nummularia* exposed to 400mM NaCl, indicating a role for H^+-ATPase in response to salt stress (Braun et al, 1986). Additionally, the NaCl responsiveness of *A. nummularia* to accumulate plasma membrane H^+-ATPase mRNA was substantially greater than in the salt-sensitive tobacco (Niu et al., 1993).

That plasma membrane H^+-ATPase may be a salt tolerance determinant was confirmed by mutation of the *AHA4* gene controlling the Na^+ flux across the endodermis (Vitart et al., 2001). Additionally, cDNA fragments corresponding to the plasma membrane H^+-ATPase from rice, designated as *OSA1*, *OSA2* and *OSA3*, are involved in the control of salt uptake into root symplast and apoplast, and further translocation in the shoot (Zhang et al., 1999).

Vacuolar ATPase (V-ATPase) acidifies intracellular compartments and contributes to the H^+-electrochemical gradient capable to drive the secondary transport of ions and metabolites across the tonoplast (Ratajczak and Wilkins, 2000). The V-ATPase holoen-

zyme has two main domains (peripheral and a membrane integral domain, Ratajczak, 2000) and three subunits: A, B and C, where subunit C transcripts increase in response to salinity (Chen et al., 2002). Increases in the tonoplast H^+-ATPase activity under salinity conditions have been reported for different species (Mansour et al., 2003), in contrast to an inhibition of vacuolar H^+-pyrophosphatase by increased NaCl concentrations (Blumwald et al., 2000). In the halophyte *Sueada salsa* the main strategy of salt tolerance seems to be up-regulation of vacuolar H^+-ATPase activity, while H^+-pyrophosphatase had a minor role (Wang et al., 2001). Nevertheless, an overexpression of the *AVP1* gene encoding the native vacuolar H^+-translocating pyrophosphatase resulted in an increased salinity tolerance in *Arabidopsis* compared with the wild-type plants (Gaxiola et al., 2001). Thus, role and activity of H^+-pyrophosphatase in responses to salinity is still uncertain.

8.2.2. Determinants Involved in Control of Na^+ Uptake and Movement – Carriers and Ion Channels

Carrier–type transport systems are characterized by exhibiting conformational changes in the transport protein. The ion-coupled transport in salinity conditions operates as symport and antiport. Carrier transport proteins act against a gradient and are energized by coupling to an electrochemical gradient, such as high affinity K^+ accumulation energized through coupling to the trans-membrane proton flux (Maathuis and Amtmann, 1999). The main carriers involved in sodium and potassium uptake and sodium influx across the plasma membrane are high affinity potassium carriers (HKT) and low affinity cation carriers (LCT) (Amtmann and Sanders, 1999, Blumwald et al., 2000). High affinity K^+ uptake is related to two gene families: 1) *KUP-HAK*, extremely selective for K^+ and blocked by Na^+ when present in mM concentrations (Santa-Maria et al., 1997, Kim et al., 1998), and 2) *HKT1* (Schachtman and Schroeder, 1994), which represents a putative pathway for high-affinity K^+ uptake and low-affinity Na^+ uptake (Maathuis and Amtmann, 1999).

Transgenic wheat lines, expressing the HKT1, exhibited enhanced growth under salinity, due to reduced Na^+/K^+ ratios when compared with the control plants (Laurie et al., 2002). In contrast to the wheat HKT1, homologous transporter from *Arabidopsis* (AtHKT1) can mediate Na^+ and, to a small degree K^+ transport in heterologous expression systems (Uozumi et al., 2000). This transporter may be involved in Na^+ recirculation from shoot to root, probably by mediating Na^+ loading into the phloem in shoots, and unloading in roots (Berthomieu et al., 2003). Similar transporters have been isolated from the japonica rice: OsHKT1 and OsHKT2, acting as Na^+ transporter and Na^+- and K^+-coupled transporter, respectively (Horie et al., 2001). Garciadeblas et al. (2003) supposed that OsHKT transporters are involved in Na^+ movement in rice, and that OsHKT1 specifically mediates sodium uptake in rice root in conditions of K^+ deficit. Additionally, OsHKT4 was be the Na^+ transporter of a low affinity. Low affinity cation carrier, encoded by *LCT1*, which was cloned from wheat (Schachtman et al.,

1997), is speculated to be important for Na^+ uptake in conditions of high salinity (Maathuis and Amtmann, 1999). The *LCT1* expression in wheat resulted in suppressed uptake of Na^+ by K^+ and Ca^{2+}, indicating that LCT1 may represent a molecular link between Ca^{2+} and Na^+ uptake into plant cells (Amtmann et al., 2001). Low affinity K^+ carriers, such as AKT1 are inward rectifying channels that activate K^+ influx and display a high K^+/Na^+ selectivity ratio, but also can mediate a significant uptake of sodium ions under high salinity (Blumwald et al., 2000).

Ion channels are distinguished form carriers by their capacity to catalyze transmembrane ion movement without conformational change. Ion channels are integral components of all membranes operating as dynamic ion transport systems coupled via membrane electrical activities (White, 1999). There are four major groups of ion channels, classified according to the gating mechanism (Krol and Trebacz, 2000): 1) ligand-gated (able to bind intracellular second messengers), 2) voltage-gated (responsible for signal transmission and transduction in response to changes in the membrane potential), 3) stretch-activated (additional transmembrane receptors) and, 4) light -activated (involved in light signal transduction). Since sodium-specific channels in plants have not been reported yet, it seems that sodium moves through the general cation channels with different permeability for particular ions (Schachtman et al., 1991, Amtmann and Sanders, 1999).

Competition between Na^+ and K^+ for intracellular influx by utilization of the common ion channels has physiological basis, since K^+ is essential co-factor of many enzymes. Sodium is an inhibitor of the enzymatic activity even in the halophytes, but at the same time it is the most available and the cheapest vacuolar osmolyte for plants in saline environments. Regarding control of sodium uptake and transport, especially from the aspect of K/Na selectivity, the voltage-gated plasmalemma K^+ channels have very important role and are classified into the inward (K^+_{in}) and outward (K^+_{out}) rectifiers. The K^+_{in} (KIRCc) and K^+_{out} (KORCs) channels are activated by hyperpolarizing potentials and by membrane depolarization, respectively, and controlled by cytosolic Ca^{2+}, ATP and pH in different ways (Grabov and Blatt, 1997).

Outward rectifiers of the root parenchyma cells are responsible for xylem loading (Maathuis et al., 1997). Salt-induced depolarization of the root plasma membrane may activate outward rectifying potassium channel, enabling the diffusion of Na^+ into the cells down its electrochemical gradient (Schachtman et al., 1991). Two classes of the outward rectifying channels have been cloned from plants: first is energized by Na^+, whereas the second class of K^+ transporters is comprised of a large gene family, expressing dual affinity for K^+ (Schachtman, 2000). Inward rectifiers are very sensitive for K^+ over Na^+ (Amtmann and Sanders, 1999). Inward rectified cation channels (which close on depolarization) are involved in salt adaptation by reducing the permeability for sodium and potassium ions, leading to decrease in the entry of Na^+ ions and the leakage of K^+ ions out of the cells under high salinity conditions (Jacoby, 1994). Sodium ion is a competitor for uptake through the K^+ inward rectifying channels at the plasma membrane, such as AKT1, which is the member of the Shaker-type family (Schachtman,

2000). In *Arabidopsis*, three of nine, so far identified genes of the Shaker K^+ channel family: *AKT1*, *AKT2* and *SKOR*, encode proteins that regulate K^+ uptake by the root, K^+ transport in the phloem tissues and K^+ secretion into the xylem sap, respectively, whereas a novel gene *AtKC1* is included in K^+ uptake from the medium (Pilot et al., 2003).

Plasmallemma voltage-insensitive cation channels (VICs) and K^+_{in} channels are responsible for an influx of sodium across the plasma membrane (White, 1999). The VIC channels are non-selective amongst monovalent and, in same cases, divalent cations and their role is related to low affinity Na^+ uptake and the stabilization of membrane potential, as well as the fast adaptation to osmotic stress (Maathuis and Amtmann, 1999). The VIC channels exhibit lower selectivity for K^+ over Na^+ than rectifying channels, thus providing the massive Na^+ entry in saline conditions over a wide range of voltages (Amtmann and Sanders, 1999).

8.2.3. Determinants of Anion Transport

Anion uptake is opposed by negative internal membrane potential, and it must be accompanied by the proton uptake to be energetically active (Michelet and Boutry, 1995). Influx of Na^+ facilitate uptake of Cl^- down the chemical gradient. Chloride is thought to traverse the root by a symplastic pathway. Anion channels regulate anion efflux from the cell through plasmalemma and the tonoplast (Krol and Trebacz, 2000). Electrophysiological and biochemical studies demonstrated the presence of an electrogenic symporter mediating the Cl^- influx and efflux across the plasma membrane, and the Cl^- antiporters are involved in the chloride transport into the vacuole (White and Broadley, 2001).

8.2.4. Determinants of Sodium Compartmentation

Accumulation of sodium in the vacuole is dependent on vacuolar H^+-translocating enzymes and tonoplast Na^+/H^+ antiporters, which are induced by saline environment (Barkla and Pantoja, 1996). An immediate effect of salt stress is vacuolar alkalization, linked with Na^+/H^+ antiporter activity of tonoplast vesicles (Hasegawa et al., 2000). The first plant Na^+/H^+ antiporter gene, exhibiting high homology with yeast antiporter NHX1, was isolated from *Arabidopsis* and designated as *AtNHX1* (Xiong and Zhu, 2002). Research studies of *Arabidopsis* antiporters confirmed their role in salinity tolerance of plants (Aharon et al., 2003).

The *Arabidopsis AtNHX* family comprises six genes, indicating that plants have significant need to regulate Na^+ homeostasis through vacuolar compartmentation, independently on the fact that sodium is not an essential mineral element (Yokoi et al., 2002*b*). Functional characterization of AtNHX members showed that AtNHX1 and AtNHX2 transcripts were widely distributed in all tissues (in difference to low abundance of AtNHX5 transcripts), whereas AtNHX3, and AtNHX4 transcripts were found in the flower and root tissues, respectively (Aharon et al., 2003). AtNHX2 and AtNHX5,

together with the AtNHX1, are the salt-tolerance determinants, acting in facilitation of Na^+ compartmentation and maintenance of intracellular K^+ status (Yokoi et al., *2002b*).

Migration pattern of antiporter gene isolated from the halophyte *Atriplex gmelini* (*AgNHX1*) correlates with H^+-pyrophosphatase, indicating its role in vacuolar compartmentation (Hamada et al., 2001). The product of the novel gene isolated from rice, the *OsNHX1*, functions as the Na^+/H^+ exchanger and plays an important role in salt tolerance (Fukuda et al., 1999). Expression of Na^+/H^+ antiporter gene found in citrus (*cNHX1*) was markedly induced by salt stress, supporting its role in salt tolerance (Porat et al., 2002).

Transcription of *NHX1* gene and a number of related genes in *Arabidopsis* was up-regulated by drought and/or salinity stress (Yokoi et al., 2002*a*), which is partially dependent on ABA biosynthesis and ABA signaling (Shi and Zhu, 2002). It seems that up-regulation of tonoplast transporters is associated with pleiotropic up-regulation of other genes, or of the activity of the gene products (Tester and Davenport, 2003). Besides the *AtNHX* family, another gene family has also been identified in *Arabidopsis* to encode the cation exchangers (*CAX* genes) responsible for modulation of ion fluxes across the vacuolar membrane (Cheng et al., 2002).

The significance of Na^+/H^+ antiporters in sodium translocation into the vacuoles has been reported for halophytic plants, such as common ice plant (Barkla et al., 2002). Nevertheless, some plants do not have Na^+/H^+ antiporters, which means that such species must rely on some other pathways and mechanisms to reduce sodium uptake, probably by utilization of K^+ channels and/or transporters that are more selective for K^+ (Blumwald et al., 2000).

The ability for harmonizing the sodium uptake, transport, sequestration and maintenance of energy pools is fundamental in salt tolerance. For better understanding of the crucial mechanisms involved in regulation of the activity of diverse channel types, the facilitating ion fluxes across the cell membranes, both in salt-sensitive and salt-tolerant species, will be major issue in the future studies on salt tolerance in plants.

8.3. Signaling Pathways of Salt Stress Tolerance

Metabolic pathways triggered by the receptor perceiving salt stress leading to the alterations in gene transcription and protein activity mediated by various signaling components, have been reviewed (Hasegawa et al., 2000, Zhu, 2000, Tester and Davenport, 2003). Candidate receptors for ionic stress could be various ion transporters. Related changes in receptor occupancy or receptor clustering may activate the ionic stress signaling pathways (Xiong and Zhu, 2002).

Mitogen-activated protein kinase (MAPK) cascade and triggering of conformational alterations in membrane proteins are known as signaling modules initiated by osmotic stress (Xiong and Zhu, 2002). Several proteins found in *Arabidopsis* are functional components of an osmotic stress MAPK cascade (Hasegawa et al., 2000). Accumulation of the reactive oxygen species (ROS), caused by salinity stress, seems to

activate the MAPK cascade, as well (Kovtun et al., 2000). Some mechanisms of signaling pathways involved in salt tolerance may be associated with stretch-activated channels and ion-specific receptors, with significant role of calcium transporters and components of Ca^{2+}-related signal transductional pathways (Tester and Davenport, 2003).

The Ca^{2+} has at least two roles in salt tolerance: 1) a pivotal participation in salt stress signaling that controls ion homeostasis pathways (Yokoi et al., 2002*a*), and 2) direct inhibitory effect on a Na^+ entry (e.g. Lazof and Bernstein, 1999). The first role was confirmed by Ca^{2+}-dependent activation of phosphatase leading to transcription of the *ENA1* gene, which encodes the P-type ATPase (Mendoza et al., 1994). Components of signal recognition and transduction pathways initiate the action of a calcium sensor on a protein kinase that affects the activity of Na^+/H^+ antiporter (Shi et al., 2000).

In *Arabidopsis*, the *SOS1* (Salt Overly Sensitive) locus is essential for Na^+ and K^+ homeostasis, as well as for the control of the long-distance Na^+ transport and loading Na^+ into the xylem under severe and mild salt stress, respectively (Shi et al., 2002*a*). SOS1 ion transporter, the SOS2 protein kinase, and its associated Ca^{2+} sensor - the SOS3 constitute a functional module (Quintero et al., 2002). The *Arabidopsis SOS2* gene, which is presumed to encode a serine/threonine protein kinase, is required for intracellular ion homeostasis (Liu et al., 2000). Although vesicles of sos2 and sos3 plants had reduced plasma membrane Na^+/H^+-exchange activity, transport ability in the mutants increased with the addition of activated SOS2 protein, indicating that SOS2 and SOS3 are involved in regulation of SOS1 transport activity (Qiu et al., 2002). Moreover, the recent discovery of *SOS4*, a novel salt tolerance determinant encoding pyridoxal kinase, pointed out the role of this gene in Na^+ and K^+ homeostasis by modulating the activities of ion transporters (Shi et al., 2002*b*). Components of SOS signal pathway, which was identified to be a pivotal regulator of plant ion homeostasis, operate in the hierarchical sequence (Hasegawa et al., 2000). This signaling pathway resembles the yeast calcineurin cascade, controlling Na^+ fluxes across the plasma membrane (Bressan et al., 1998).

According to Zhu (2000), the SOS pathway for plant Na^+ tolerance includes: a) high salt stress leading to an increase in cytosolic free Ca^{2+} concentrations, b) binding of SOS3 to Ca^{2+} which activates the protein kinase SOS2, c) activated SOS3-SOS2 kinase complex increasing the expression of *SOS1* and probably other transporter genes under salt stress, and d) this gene expression (remarkable also at the post-translation level) and regulation of transporter activity leading to ion homeostasis and, thus enhancing salt tolerance in plants.

Majority of Ca^{2+}-stimulated protein phosphorylation is performed by members of the Ca^{2+}-dependent protein kinase (CDPK) family (Sanders et al., 1999). Many of transport proteins, such as membrane transporters, H^+-ATPases and aquaporins involved in osmoregulation of the cell, are regulated by CDPKs (Li et al., 1998). Gene found in rice that encodes a Ca^{2+}-dependent protein kinase (designated as *OsCDPK7*) was induced by salt stress, maintaining the signaling pathway by unknown post-translation mechanism (Saijo et al., 2000). The GSK3/shaggy-like protein kinases play an

important role in signal transduction, as was reported for *Arabidopsis AtGSK1*, whose overexpression in transgenic plants was seen in response to salinity conditions (Piao et al., 2001).

Exogenous ABA can activate transcription of group of genes induced by salt or water stress, while other stress-inducible genes were not activated, suggesting ABA-dependent and ABA-independent signaling pathways (Shinozaki and Yamaguchi-Shinozaki, 1997). There is information on ABA-dependent transcriptional activation related to Ca^{2+}-dependent protein kinases (CDPKs) (Sheen, 1996), and ABA-independent transcription factors such as dehydration response element (DRE) binding proteins (Liu et al., 1998). The ABA-dependent and ABA-independent expression pathways were also obtained in the case of the LEA gene *Dc3* from carrot in transgenic tobacco (Siddiqui et al., 1998). The expression of osmotically induced gene *saltT* in rice is initiated by either salinity or ABA through antagonistic signal transductional pathways (Garcia et al., 1998). Drought and salt stress induce ABA biosynthesis largely through transcriptional regulation of ABA biosynthesis genes (Xiong and Zhu, 2003).

8.4. Detoxification and ROS Scavenging

Oxidative stress is an additional phenomenon of stress impact on plants. This secondary effect emerges as a consequence of hyperosmolarity caused by imposing of plants to drought or salt stress conditions, resulting in appearance of the reactive oxygen molecules, such as hydrogen peroxide, hydroxyl radicals and superoxide anions (Xiong et al., 2002). The scavenging of reactive oxygen species (ROS) is associated with both activity of the enzymes involved in antioxydative processes of the cell (particularly superoxide dismutase, glutathione peroxidase and catalase) and the presence of osmoprotectant compounds, including mannitol and proline (Xiong et al., 2002). Harmful influence of ROS on cell macromolecules may be also alleviated by the activity of antioxidant compounds such as ascorbic acid, glutathione, thioredoxin and carotenoids (Xiong and Zhu, 2002). Preincubation of particular enzymes *in vitro* with a various osmolytes prevented a salt-induced inhibitory effects on the enzyme activity (Ghosh et al., 2001). Participation of antioxidative system in responses to salt stress was studied in tomato and its wild salt-tolerant relative *Lycopersicon pennellii* (Shalata and Tal, 1998). Activities of superoxide dismutase (SOD), ascorbate peroxidase (APX) and dehydroascorbate reductase (DHAR) under concentration of 100 mM NaCl were inherently higher in *L. pennellii* than in *L. esculentum*.

Free radical-mediated damage of membrane might be a component of the cellular toxicity of salt-stressed rice seedlings, according to the ability of the salt-tolerant varieties to maintain the specific activity of antioxidant enzymes (SOD and peroxidase), in difference to salt-sensitive varieties (Dionisiosese and Tobita, 1998). Higher capacity for ROS scavenging was observed in tomato calli tolerant to 50 mM NaCl in comparison with control, on the basis of the activity of lipoxygenase and antioxidant enzymes (Rodriguezrosales et al., 1999). Exposure of wheat genotypes to long-term salt stress

has caused an increase of the activity of superoxide dismutase, catalase and glutathione reductase, which was more obvious in the salt-tolerant varieties (Sairam et al., 2002). Rapid increase in the activity of ascorbate peroxidase, catalase, glutathione reductase, peroxidase and, especially superoxide dismutase observed in salt-treated cotton callus, suggests the importance of the up-regulation of antioxidant capacity in an early response to salt stress (Manchandia et al., 1999). The findings concerning the increased activity of antioxidant enzymes of salt-tolerant varieties in response to salt stress imply that ROS scavenging might be a part of the general adaptive strategy of plants exposed to salinity. Expression of many genes is regulated by ROS, especially of those involved in encoding the ROS scavengers, either enzymes or antioxidants (Jamieson, 1998).

Several genes which encode the antioxidant enzymes have been isolated and characterized. Examples are *csa* gene of citrus cells corresponding to phospholipid hydroperoxide glutathione peroxidase (Avsiankretchmer et al., 1999), and the *CAT2* and *Call* genes found in *Nicotiana plumbaginifolia* responsible for NaCl-induced catalase activity (Savoure et al., 1999). Overexpression of the tomato *TPX2* gene and *swpa1* gene from sweet potato in transgenic plants contributed to salt tolerance or oxidative-stress tolerance, respectively (Yoshida et al., 2003). In an experiment with salt-adapted and unadapted tomato cells, adapted cell suspension has exhibited the higher threshold concentration for down-regulated *TPX1* (peroxidase encoding gene) transcripts (Medina et al., 1999). Differential gene expression of superoxide dismutase isoforms in rice exposed to drought, chilling and salinity showed that phytohormone (ABA) and active oxygen species are associated with the regulation of SOD genes under environmental stresses (Kaminaka et al., 1999).

Complex network of adaptive reactions and interactions at physiological and molecular level, involving the coordinated interaction of many salt-inducible and regulatory genes, gene products and signaling athways, permits survival and growth of plants exposed to salinity. This is through the two main strategies referring to more or less restricted salt uptake and accumulation evolved in halophytes and non-halophytes, respectively (Figure 5).

9. GENETIC ASPECTS OF SALT TOLERANCE AND ADVANCES IN BIOTECHNOLOGY

The salt-adaptive capacity of species may be related to constitutive expression of genes that encode salt-tolerance determinants (Casas et al., 1992) or the greater capacity to regulate the expression of these genes in response to salt (Cushman et al., 1990*b*). Salt-inducible genes fall into different categories, as per their function, predicted from sequence homology with known proteins, such as osmoprotectants and molecular chaperones, proteins involved in ion transport, signaling and oxidative stress defense systems, proteinases, transcriptional factors, as well as proteins controlling photosynthesis and other physiological processes.

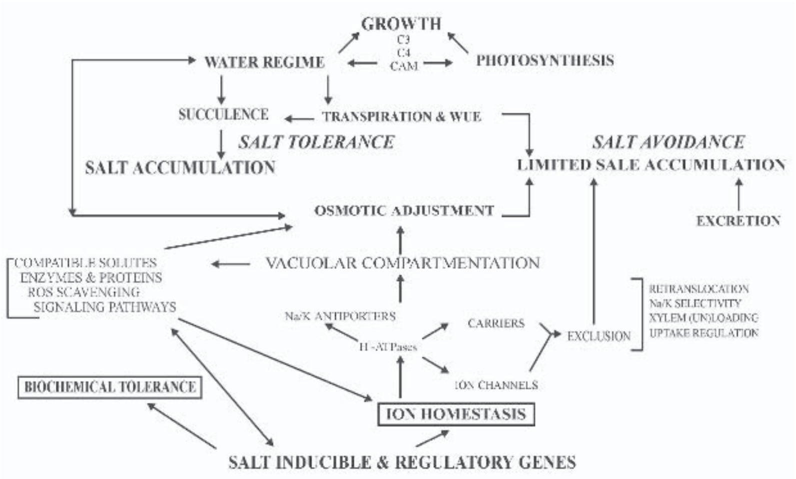

Figure 5. *Determinants of salt tolerance related to the main adaptive strategies (salt tolerance and salt avoidance) of plants exposed to salinity*

A survey of cell responses to salt stress identified a large number of genes induced by salt. For example, 218 cDNA salt-inducible clones have been detected in barley roots, of which 133 cDNA clones have homology to known proteins and 24 are identified as genes for signal transduction (Ueda et al., 2002). A recent salt-treated cDNA library of the halophyte *Suaeda salsa* can serve as a useful model for research in salt-tolerant plants (Zhang et al., 2001). Differential display in mangrove *Bruguiera gymnorrhiza* revealed nine transcripts up-regulated or induced by salt stress (Banzai et al., 2002). The gene for NADP-malic enzyme in *Aloe vera* (*AvME*) was identified as a gene induced by salt stress, whose expression was related to the degree of salt tolerance (Sun et al., 2003). Five cDNAs isolated from wheat by modified differential display were named *WESR* (wheat early salt-stress responding) and described as novel salt-stress responding genes (Nemoto et al., 1999). Mutational analysis of salt-sensitive *Arabidopsis* to characterize salt-induced genes has been very useful in identifying as well as their signaling pathways were determinants of salt tolerance (Zhu, 2000). The coordinate mRNA accumulation induced in salt stress conditions has been reported in different plants (Bohnert et al., 1995; Bray, 1997).

Alterations in transcript levels of many gene families in response to cation stress revealed the existence of putative non-selective cation channels for NaCl treatment, metal transporters for Ca^{2+} starvation conditions, and the changes in mRNA levels related to primary pumps, antiporters and aquaporins induced by stress (Maathuis et al., 2003). As per the transcriptome changes in *Arabidopsis* in response to salt, osmotic and cold stress, about 30% of the transcriptome is sensitive to regulation of common stress conditions, and majority of changes were stimulus specific (Kreps et al., 2002).

The small molecules designated as transcription factors bind to promoter regulatory elements in salt-inducible genes, whose role is associated with the transcriptional activation of genes regulated by salt and drought stress (Shinozaki and Yamaguchi-Shinozaki, 1997). An altered gene expression in salt-tolerant alfalfa seems to be connected with the involvement of a putative transcriptional factor Alfin1, while overexpression of the *Alfin1* in transgenic alfalfa led to the activation of an additional salt-inducible gene, the *MsPRP2* (Winicov, 1998), suggesting a role of transcription factors in coordinate gene regulation under salinity conditions.

Salinity tolerance has been considered to be a quantitative trait (Foolad and Jones, 1993). Hence, the identification of quantitative trait loci (QTL) that contribute to natural variation in responses to salt stress will be helpful in understanding the complexity of the genetic control of salt tolerance and provide opportunities for more efficient breeding for salt tolerance in crops. In the study of quantitative trait loci controlling various traits of rice seedlings exposed to salinity stress, out of a total of seven QTLs for traits associated with improved tolerance, four were located on chromosome 6 (Prasad et al., 2000*a*). It was reported that QTLs for sodium and potassium uptake in rice were found on different chromosomes (Koyama et al., 2001), which is consistent with the independent inheritance of Na^+ and K^+ uptake in the mapping populations, and with different uptake pathways (apoplastic leakage and membrane transport, respectively) for these cations under saline conditions. In rice, the putative locus for osmotic adjustment and two of five QTL associated with dehydration tolerance were close to chromosomal regions responsible for the root morphology (Lilley et al., 1996).

Identification of QTLs for salt tolerance during vegetative growth in tomato led to detection of five genomic regions on chromosomes 1, 3, 5, 6, and 11 (Foolad et al., 2001). In *Arabidopsis,* 11 QTLs were detected, for variation in salt tolerance, of which 6 were present during germination and 5 at the vegetative growth stage (Quesada et al., 2002). A survey of QTLs for salt tolerance in barley seedlings revealed 12 quantitative trait loci for seven traits (Ellis et al., 2002). A composite genomic map for the *Triticeae* of QTLs affecting stress tolerance showed the highest concentration of QTLs and major loci controlling the plant's adaptation to environmental stresses located on the chromosome group 5 (Cattivelli et al., 2002). The QTL analysis performed with 172 recombinant hybrids between *Helianthus annuus* and *H. petiolaris*, revealed a total of 14 QTLs for mineral uptake and three for survival, suggesting that salt tolerance in *Helianthus* is achieved through increased calcium uptake, coupled with greater sodium exclusion (Lexer et al., 2003). It was reported for several species that QTLs linked to salt tolerance vary with the developmental stage of the plant (Flowers, 2004).

Significant progress in highlighting the physiological and biochemical responses of plants to salinity at different levels, as well as the molecular cloning of genes involved in the different metabolic pathways altered by salts, has enabled the introduction of genetic engineering technologies in order to improve salt tolerance in crops.

9.1.Transgenic Plants

Plant genetic engineering, which began in the mid-eighties, is at present much focused on the genetic responses of plants to abiotic stresses, including salinity stress. To pyramid useful genes for high-level resistance of plants to adverse effects of the environment, is still a challenging. While genes of many functional groups related to salinity stress are being broadly surveyed, a group of genes has been recently tested in transgenic plants for their effect on salt tolerance. There are reports on involvement of genes of transgenic plants in tolerance against abiotic stresses (Grover et al., 1998, Altman, 2003) and possibilities for both elucidating the roles of new stress responsive genes and engineering the whole cascade of multiple genetic changes through manipulation of the regulatory genes (Grover et al., 1999).

Regarding biotechnological strategies to enhance plant salt tolerance, much effort has been made in the field of oxidative stress responses, regulation of ion homeostasis, signaling pathways and, especially osmoprotectant synthesis (Bohnert and Shen, 1999, Rathinasabapathi 2000, Yoshida, 2002, Sakamoto and Murata, 2000, Apse and Blumwald, 2002). Many major crops lack the required capacity for synthesis of compatible solutes, which are naturally accumulated by stress-tolerant species. Transformation of tobacco with the *Escherichia coli* genes for glycine betaine synthesis (*betA* and *betB* genes) resulted in transgenic plants accumulating glycine betaine, and which exhibited increased salt stress tolerance and enhanced recovery from photoinhibition caused by salinity (Holmström et al., 2000).

Installing the gene for synthesis of choline monooxygenase (*CMO*) from the halophyte *Suaeda liaotungensis* in tobacco led to accumulation of betaine in transgenic plants, which were able to survive on medium containing 250 mM NaCl (Li et al., 2003*b*). Similar results were obtained with the betaine aldehyde dehydrogenase gene (*BADH*) from *S. liaotungensis* in transgenic tobacco (Li et al., 2003*a*). The *BADH* gene cloned from *Atriplex hortensis*, a natural glycine betaine accumulator, when transformed into a salt-sensitive tomato cultivar, contributed to successful growth of transgenic plants at 120mM NaCl (Jia et al., 2002), and overexpression of the *CMO* gene isolated from the same donor *A. hortensis* (*AhCMO* gene), in tobacco resulted in better performance under both salt and drought stress (Shen et al., 2002). Expression of the *BADH* gene from sorghum, in hairy roots of transgenic tomato contributed to the maintenance of the osmotic potential under salinity treatment (Moghaieb et al., 2000). The *codA* gene for biosynthesis of glycine betaine from *Arthobacter globiformis* when installed into *Brassica juncea*, led to an enhanced capacity for seed germination and improved seedling growth in saline media (Prasad et al., 2000*b*).

Despite the fact that salinity tolerance of plants is a multigenic trait, most of the research with transgenic plants showed that transfer of a single or a few genes resulted in an increase in salt tolerance, but which has to be tested at the whole plant level (Flowers, 2004). Some examples of improved salt tolerance of transgenic plants are presented in Table 4.

***Table 4**. Some examples of genetic transformation resulting in enhanced salt tolerance*

Gene	*Function*	*Origin*	*Transgenic plant*	*Reference*
Atcys-3A	cysteine synthesis	*Arabidopsis*	yeast	Romero et al., 2001
AgNHK1	Na/H antiporter	*Atriplex gmelini*	rice	Ohta et al., 2002
AtNHX1	Na/H antiporter	*Arabidopsis*	yeast	Aharon et al., 2003
HKT1	K transporter	wheat	Florida wheat	Laurie et al., 2002
AtHKT1	Na/K transporter	*Arabidopsis*	yeast	Uozumi et al., 2000
HAL1	K/Na selectivity	Yeast	tomato	Rus et al., 2001
RS domains	splicing proteins	*Arabidopsis*	yeast	Forment et al., 2002
AhDREB1	DNA binding protein	*Atriplex hortensis*	tobacco	Shen et al., 2003
BveIF1A	translation initiation	sugar beet	*Arabidopsis*	Rausell et al., 2003
DsALDP	fructose-1,6-diphosphate aldolase	*Dunaliella salina*	tobacco	Zhang et al., 2003

10. BREEDING FOR SALINITY TOLERANCE AND IMPROVING SALT TOLERANCE OF PLANTS

Although plant breeding for improved salt tolerance in crops can't be the only approach in resolving the problem of increasing salinity throughout the world, it should be considered as part of a total package (together with the management of irrigation and drainage, as well as application of proper agricultural practices) which aims to utilize the salt-affected land in a sustainable way. Resistance to saline conditions can be increased beyond the existing phenotypic range by selecting individual physiological traits contributing to salt tolerance and combining them in breeding programs (Khatun et al., 1995).

There are several ways of dealing with the problem of salinity, as indicated by Flowers and Yeo (1995), such as: direct use of halophytes, (relatively cheap method of domestication of salt-tolerant plants) incorporation of genetic information for salt

tolerance from halophytic species via wide-hybridization and single gene transfer through genetic engineering, improvement within existing crop genomes by screening and breeding based on natural genetic variability, use of mutants and cell and tissue culture (to generate novel genotypes) and breeding only for yield potential and yield stability.

Conventional breeding techniques (such as interspecific hybridization, screening and recurrent selection) rely on existing genetic variability of plants in responses to salinity. There are many reports referring to screening for salt tolerance of varieties, genotypes and lines of crops, such as barley (Wei et al., 2003), rice (Zeng et al., 2003), wheat (Saneoka et al., 1999, Rivelli et al., 2002*a*), sugarcane (Plaut et al., 2000), soybean (Essa, 2002, An et al., 2002), citrus (Moya et al., 2002), canola (Qasim et al., 2003), grapevine (Storey et al., 2003), bean (Bayuelo-Jimenez et al., 2002) and alfalfa (Al-Khatib et al., 1993).

The International Rice Research Institute (IRRI) has developed a number of salt-tolerant varieties in rice. (Moeljopawiro and Ikehashi, 1981). High- and low-sodium transporting lines of rice were developed by intravarietal selection (Yadav et al., 1996). Four rice varieties which were tested at the National Centre for Genetic Engineering in Bangkok have been chosen for further tests, as they expressed an ability to survive irrigation with water containing 2% to 3% NaCl (Öko- Institut, http://www.plantstress.com/Articles/up_general_files/GE_Tol.pdf). Breeding for salt tolerance carried out at the Central Soil Salinity Research Institute (CSSRI, Karnal, India) resulted in the development of CSR10, the first salt-tolerant early maturing rice variety of the country, capable to withstand highly alkaline and saline conditions (EC_e of up to 10 dS m^{-1}) under transplanted irrigated management system (http://www.plantstress.com/admin/files/Salt_Karnal.htm). Furthermore, CSSRI has released several other salt-tolerant varieties, including two fine and one superfine grain rice variety, two wheat varieties and one of Indian mustard.

For certain crops, wild relatives can offer higher level of salt tolerance and help in wide hybridization. Several wild relatives of tomato were tested and evaluated for salt tolerance, including *Lycopersicon pimpinellifolium*, *L. cheesmanii*, *L. peruvianum* and *L. pennellii* (Cuartero et al., 1992). Recurrent selection for salt tolerance of the interspecific hybrids resulting from backcrosses to a domestic tomato cultivar, led to the appearance of plants able to survive in up to 70% of the seawater salt concentration (Subbarao and Johansen, 1994). Interspecific crosses between species of the same genus had certain success in improving salt tolerance within some cross-pollinating species, such as rice, wheat, tomato and barley (Biosalinity Awareness Project, http://www.biosalinity.org/halophytes.htm).

In spite of high expectations, conventional breeding programs led so far, to only about 30 cultivars in just 12 with improved salt tolerance (Flowers and Yeo, 1995, Flowers, 2004). This may be associated with extremely difficult and time consuming testing of salt tolerance of crops in the field, due to: a) enormous ability of salt-affected soil to interact with other environmental factors, b) changeable salinity within fields

and during the vegetation season, and c) an altered sensitivity to salt stress depending on the developmental stage of a plant (Flowers, 2004).

Cluster group ranking of genotypes based on multiple agronomic characters may be applied in breeding for salt tolerance (Zeng et al., 2002), and the method has advantages over conventional methods. According to Garcia et al. (1995), selection for agronomic traits should be made after selection for salt resistance and, ideally, delayed until the population has reached near-homozygosity. Moreover, certain improvements concerning the possibility of acclimation of crops to salinity stress have been reported for soybean pretreated with 34 mM NaCl at the seedling stage, resulting in an increased survival rate at 137 mM NaCl until maturity (Umezawa et al., 2000). Similar enhancement was reported for sorghum supplemented with 150 mM at the seedling stage, which resulted in plants able to withstand a concentration of 300 mM NaCl and even to produce seeds (Amzallag et al., 1990).

Cell and tissue culture can be applied to breeding for salt tolerance by both screening of material *in vitro* and in the generation of novel genotypes through somaclonal mutation (Flowers and Yeo, 1995). Studies with NaCl-adapted and unadapted cells in suspension culture have demonstrated that particular salt-tolerance determinants can be obtained, such as K^+/Na^+ ratio and proline accumulation in tobacco (Okuma et al., 2002) and rice cell lines (Shah et al., 2002). Somatic embryogenesis through callus initiation on high salinity levels may be a valid method for screening and selection for salt tolerance in wheat cultivars (Zair et al., 2003) and strawberry clones (Dziadczyk et al., 2003) after application of *in vitro* selection pressure. However, despite the promises during the 1980s, the use of cell culture selection for enhanced salt tolerance did not release any cultivar capable of growing successfully in farmers' fields (Flowers, 2004). Therefore, it would be necessary to compare reported increases in salt tolerance under *in vitro* conditions with results from testing these plants in the field, where increased salt tolerance is more difficult to prove.

The main approaches for improving salt tolerance of crops require backcrossing into cultivars and precise screening of progeny by use of either a quantitative trait or a molecular marker for that trait (Munns et al. 2002). Enhanced salt tolerance in plants may be achieved by the pyramiding of physiological traits, (dependent on genotypic information) or of direct knowledge of the candidate genes (Flowers et al., 2000). A detailed insight into genetic information concerning the variability within species and cultivars may provide a basis for improving general salt tolerance in plants, and therefore screening for various traits in responses to salinity of the existing genetically diverse material might be recommended as the most simplest and very convenient way.

11. SCREENING OF PLANT RESPONSES TO SALINITY, CURRENT TECHNIQUES AND METHODOLOGIES

Screening methodologies in both field and controlled conditions are based upon salinity tolerance criteria, such as: germination, survival, leaf damage, growth stage re-

sponse and yield (Subbarao and Johansen, 1994). In a recent study, on canola, field pea, dry bean and durum wheat, several agronomic parameters have been evaluated for their effects on salt tolerance, (Steppuhn et al., 2001). As screening for salt tolerance based on visible symptoms (leaf necrosis, abscission and chlorosis) is appropriate only for sensitive crops, and the classical method based on yield change is expensive, several indirect parameters and related methods, such as chlorophyll fluorescence and leaf bioelectric activity have been proposed (Shabala et al., 1998). Screening methods for salt tolerance are grouped into the following categories (Munns and James, 2003): a) methods based on growth (e.g. root and leaf elongation, biomass) and yield under controlled conditions, including both short-term and long-term experiments; b) methods of tolerance to high salinity levels, such as germination and plant survival, leaf injury, premature loss of chlorophyll and measurements of chlorophyll fluorescence; and, c) methods based on physiological mechanisms and specific traits, such as Na^+ exclusion, K^+/Na^+ discrimination and Cl^- exclusion, as well as Na^+/Ca^{2+} selectivity (Zeng et al., 2003).

Based upon the differences in physiological traits, several methodologies have been developed, especially for the purposes of analyzing the ion concentration and localization within particular tissues and cell compartments. In this category, the benefits of microelectrode measurements for studying of single cell metabolism have been advocated (Miller et al., 2001). Ion activities in living plant cells are recorded by patch-clamp techniques (White et al., 1999), fluorescent dyes (Mühling and Läuchli, 2002*b*, Halperin and Lynch, 2003) and NMR technologies (Olt et al., 2000, Gruwel et al., 2001), whereas X-ray microanalysis offers an opportunity for quantifying the amount of an element (Flowers and Hajibagheri, 2001). The studies of nutrient status and transport on the microscale rely on other methodologies as well, such as: the kinematic growth analysis and elemental deposition rates, microdissection, specimen preparations, secondary ion mass spectrometry, and other microanalytical techniques, like microautoradiography (Lazof and Bernstain, 1999). Since the extrapolation of results obtained under highly controlled conditions to real field situation has had limited success, it was necessary to develop the new and simpler field screening methods and models to enable more efficient breeding for salinity tolerance (Isla et al., 1997).

12. MODELING FOR YIELD ESTIMATION

There is no unique parameter relating crop yield to the average soil salinity, since crop growth changes with soil water deficit and other soil features. There are mathematical models for estimation of crop yield under saline conditions dealing with the physics of water movement through soil, plant and atmosphere (Grant et, al., 1993). The model named *ecosys* was expanded to include an ion transfer-equilibrium-exchange model used to calculated electrical conductivity and soil osmotic potential, and it was proposed for general use in assessing salinity effects on crop growth and water use on different soils and environmental conditions (Grant, 1995). Some other models propose

the application of static and dynamic salinity stress indices (SSI) in order to determine root zone salinity and ion flux to the shoot, respectively (Dalton et al., 1997), and the separation of physical and biochemical processes governing plant salt tolerance (Dalton et al., 2000).

The conceptual models, which do not neglect solute reactions in the root zone, the surface evaporation and influence of immobile wetted pore space can accurately predict the leaching requirements (Lr) for crops from salinity of irrigation water and the crop salt tolerance threshold (Alsaeedi and Elprince, 1999). The application of a sprinkler (such is triple line source sprinkler system) and a drip irrigation system was successfully used in screening for salinity tolerance in barley (Isla et al., 1997). Several other models dealing with estimation of specific parameters of soil salinity, two- and three-dimensional equilibrium solute transport, field-scale spatial salinity patterns, etc., have been proposed during the last twenty years by USDA (USDA Salinity Laboratory, http://www.ussl.ars.usda.gov/modelsmenu.htm). Among them, the newest model (“WATSUIT”), developed in 2001, predicts the salinity, sodicity and the toxic solute concentrations of the soil water within a simulated crop root zone. This model allows an evaluation of crop salinity threshold, slope and yield at given salinity level. Nevertheless, in case of increasing and variable salinity within farmer fields, an estimated yield for the most of the cultivated species is far from acceptable. Hence, it seems that the only reasonable option for utilization and management of highly salinized soils should be domestication and use of halophytes.

13. POTENTIAL OF HALOPHYTES IN SALINE AGRICULTURE

For extremely salt-affected soils the use of halophytes is recommended (O’Leary, 1994), since any attempt either of soil reclamation or introduction of genetically modified crops, possibly adapted to such conditions, would be enormously expensive. Therefore, the domestication of some of the more than 1500 species of halophytes quoted, seems likely to be more successful and cheaper than the long-term process of modifying and breeding of an existing crop to fit into the niche of halophytes (Flowers and Yeo, 1995). Many halophytes have great potential for use in so called ”saline agriculture”, i.e. as oil seeds, food, fodder, fuel, fiber and other products (Table 5), in addition to their possible utilization in reclamation (phytoremediation) of highly salinized soils, referred to previously.

Table 5. *Possible utilization of halophytes in saline agriculture*

Food	*Fuel*	*Fodder*	*Other Products*
Grains and oilseed	**Trees and shrubs**	**Grasses**	**Essential oils**
Zostera marina	*Prosopis spp.*	*Leptochloa fusca*	*Pandanus fascicularis*
Ditichlis palmeri	*Eucalyptus spp.*	*Paspalum vaginatum*	*Mentha sp.*
Sporobulus airoides	*Casuarina spp.*	*Salsola iberica*	*Chamomilla recutita*
Pennisetum typhoides	*Rhizophora spp.*	*Distichlis spicata*	*Vetiveria zizanioides*
Chenopodium quinoa	*Melaleuca spp.*	*Echinochloa turnerana*	*Ocimum kilimands charicum*
Kosteletzkya virginica	*Tamarix spp.*	*Spartina alterniflora*	*Cymbopogon nardus*
Acacia spp.	*Acacia spp.*	*Chloris gayana*	**Gums, oils and resins**
Terminalia catappa	*Pongamia pinnata*	*Agropyron elongata*	*Sesbania bispinosa*
Salicornia spp.	*Butea monosperma*	*Sporobulus airoides*	*Grindelia spp.*
Tuber and foliage	**Liquid fuels**	*Puccinellia distans*	*Larrea tridentata*
Batis maritima	*Beta vulgaris*	*Hedysarum* **carnosum**	*Sapium sebiferum*
Sesuvium portulacastrum	*Nypa fruticans*	**Shrubs**	*Chrysothamnus nauseosus*
Portulaca oleracea	**Gaseous fuels**	*Atriplex spp.*	*Simmondsia chinensis*
Crithmum maritimum	*Leptochloa fusca*	*Mairiena spp.*	**Pulp and fiber**
Atriplex triangularis		*Kochia spp.*	*Phragmites spp.*
Eleocharis dulcis		*Halosarcia spp.*	*Juncus spp.*
Suaeda maritima		**Trees**	*Pandanus tectorius*
Fruits		*Acacia spp.*	*Hibiscus spp.*

Table 5. *Continued...*

Salvadora spp.		*Leucaena leucocephala*	*Stipa tenacissima*
Manilkara hexandra		*Prosopis spp.*	*Typha domingensis*
Santalum acuminatum			*Gossypium hirsutum*
Coccoloba uvifera			Palms
Lycium fremontii			**Bioactive derivates**
Leaf protein			*Calophyllum inophyllum*
Kochia scoparia			*Balanites spp.*
Salsola kali			*Catharanthus roseus*
Beta maritima			*Adathoda vasica*
Salicornia spp.			mangrove species
Atriplex spp.			**Ornamental use**
Traditional crops			*Eucaliptus spp.*
asparagus			*Tamarix spp.*
barley			*Conocarpus erectus*
wheat			*Limonium spp.*

(adapted after BOSTID report, 1990)

14. FURTHER PROSPECTS AND GOALS IN RESEARCH ON PLANT SALT TOLERANCE

In summary, the improvements in plant salt tolerance can be identified by: a) screening for appropriate diversity in responses to salinity among cultivars or related wild species (Flowers and Yeo, 1995); b) treatment with mutagens in order to produce mutants which show hypersensitive or reduced responses to salinity as compared with the wild type (Zhang et al., 1995, Wu et al, 1996, Maggio et al., 2001), and c) engineering transgenic plants expressing one or more foreign genes which are expected to increase cellular resistance to salinity (Bohnert and Jensen, 1996, Neumann, 1997).

Conventional methodologies of genetic manipulation in breeding for salt tolerance have been discussed (e.g. Yeo, 1998, Munns et al., 2002). These include quantitative trait locus (QTL) analysis, introgression of whole or parts of chromosomes,

monitored with fluorescence *in situ* hybridization, as well as the pedigree selection breeding, mass selection, backcross breeding, recurrent selection and wide hybridization. The improvement of crops by investigation of favorable alleles existing in wild relatives of crops, provides a potential opportunity for achieving advances in crop performance, as well as screening for a range of traits for salt stress tolerance, within large populations of mutant plants (Miflin, 2000).

New molecular marker technologies can be used for marker-assisted selection (MAS) to improve the salinity tolerance of crops (Tanksley et al., 1989). Markers tightly linked to salt tolerance can be used in MAS after evaluation of their reliability. The use of molecular markers is needed for clarifying the number, chromosomal locations and genetic contributions of genes controlling both the quantitative or complex traits and simply-inherited traits under stress conditions (Lilley et al., 1996).

Since the various mechanisms and adaptive responses of plants to salt stress are multigenic traits, further efforts are necessary to comprehend the gene expression for groups of functionally related genes. In order to extend the application of gene transformation to abiotic stresses it is important to gather information on what are the "useful genes", responsible for better stress tolerance (Grover et al., 1998). Three approaches are commonly used in identification of genes responsible for salt tolerance: a) analysis of genes involved in processes associated with salt tolerance, b) identification of genes whose expression is dependent on salt stress, and c) survey based on salt tolerance determinants based on functionality (Borsani et al., 2003).

Several genetic model systems, apart from the favorite subjects of stress studies, like tomato and tobacco, offer excellent opportunities for research in salt tolerance. Examples are the common ice plant, *Arabidopsis*, yeast, recently found halophyte *Thellungiella halophila* (Zhu, 2000, 2001) and salt-tolerant green alga *Dunaliella salina* (Cowan et al., 1992).

Improved salt tolerance may be achieved by the maintenance, activation, and enhanced function of physiological systems that are especially sensitive to disruption by increased levels of salts (Winicov, 1998). On the other hand, an opposite approach could consider the favoring and improvement of those systems exhibiting distinct tolerance to salinity.

Plant responses to salt stress are complex, extremely variable, mutually linked, and include a wide range of effects at the molecular, cellular, tissue and whole-plant level. It is therefore unlikely in the near future, to have simple answers and solutions related to the problem of salt tolerance. However, there is hope that many aspects of salt stress would be resolved, particularly through the knowledge from molecular biology, biotechnology and bioinformatics. Further, knowledge on salt-inducible genes, genetic control of salt responses and signaling pathways offers a chance for creating a clearer picture of plant responses and adaptations to salinity.

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CHAPTER 4

HIGH TEMPERATURE STRESS

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1. INTRODUCTION

The global mean temperature increased by 0.6°C between 1990 to 2000, and is projected to increase by another 1.4 to over 5°C by 2100 (Houghton et al., 2001; McCarthy et al., 2001). Plants suffer the ups and downs of temperature of their environment, while animals often regulate their temperature, either by movement or metabolism. Therefore, global warming may affect plants more than animals and there are indications that plants experience substantial damage from high temperature stress. Estimates range up to a 17% decrease in crop yield for each degree Celsius increase in average growing season temperature (Lobell and Asner, 2003).

While the temperature of plants is strongly dependent on ambient air temperature, it is also dependent on radiant energy fluxes. Almost all crop plants, and most plants in nature experience the full intensity of sunlight for at least part of their lives. To fully understand heat stress effects on plants it is necessary to know what temperatures plants experience; this is analyzed by energy balance equations. At equilibrium, energy gain and loss is constant and heat gain by one process is balanced by heat loss by another. Three important routes of heat gain or loss are (1) sensible heat transfer, (2) radiant heat transfer, and (3) latent heat transfer (e.g., evaporative cooling). Typically, a photosynthesizing leaf is being heated by radiant heat gain and losing energy by sensible heat loss and latent heat loss, but each of these factors can be negative or positive.

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1.1. Sensible Heat Transfer

Heat will flow to or from a plant depending on the difference in temperature between the plant and its environment. This heating or cooling is intuitive; whenever a plant is above air temperature it will lose heat to the air, if it is below air temperature it will gain heat. The rate of transfer will depend on the wind speed because of the boundary layer between the leaf and the well-mixed air. Small structures like stems and small leaves have low boundary layer resistances to heat flow, while large leaves can have large boundary layer resistances to sensible heat exchange with the air.

1.2. Radiant Heat

Radiant heat gain is responsible for most of the stressful high temperatures experienced by plants. Because plants photosynthesize, many plants are optimized to receive sunlight, which will also optimize radiant heat gain. Many leaves are very thin, optimizing the ability to intercept photosynthetically active radiation but also reducing the heat capacity of the leaf. This makes leaves susceptible to very rapid temperature changes. If all of the energy in sunlight were absorbed by a thin leaf (e.g., 0.4 mm^2 with 50% of the leaf being airspace and 50% being liquid phase) then the following equation applies:

$$\frac{0.1367\ \text{J}\quad {}^{\circ}\text{C g}\quad \text{cm}^3}{\text{cm}^2\ \text{sec}\quad 4.184\ \text{J}\quad \text{g}\quad 0.02\ \text{cm}} = 1.1^{\circ}\text{C sec}^{-1}. \qquad [1]$$

In words, if a thin leaf had no mechanisms for losing heat, strong sunlight could cause that leaf to boil in less than 2 min. Obviously, heat loss by leaves is an important consideration.

The energy in sunlight is often divided into two parts: (1) ultraviolet, visible, and near infrared (wavelengths between approximately 280 nm to 4 µm) and (2) thermal wavelengths (greater than about 4 µm). Very hot objects like the sun emit nearly all of their radiant energy in the visible and near infrared regions of the electromagnetic spectrum, while objects with temperatures of plants and their surroundings emit radiant energy in thermal wavelengths (Nobel, 1999). Infrared radiation exchange of a leaf is estimated by the Stefan-Boltzman law:

$$IR = a \bullet \sigma \bullet T^4 \qquad [2]$$

where a is the absorptivity (or emissivity for emission) for infrared energy, σ is the empirical Stefan-Boltzman constant ($5.67 \bullet 10^{-8}$ W m^{-2} K^{-4}) and T is temperature in

Kelvin. A substantial amount of energy can be lost by infrared emission from leaves but leaves also receive infrared energy from their surroundings. If the surroundings are the same temperature as the leaf, the net energy flux will be zero. However, on a sunny day with no clouds, the leaf is emitting infrared radiation to the sky and receiving infrared radiation from the sky. The effective temperature of the sky on a clear day can be less than 0°C and so the radiation balance in the thermal infrared wavelengths can dissipate over one half of the sunlight energy absorbed by leaves. The worst situation occurs on a partly cloudy day if the sun shines through a gap in the clouds. Clouds have a much warmer effective temperature than does the clear sky and so the energy gained from the sunlight is not as easily dissipated through infrared energy loss. The energy plants receive from sunlight beyond what is dissipated by thermal radiation must be dissipated by a combination of sensible heat loss (see above) and latent heat loss (see below).

1.3. Latent Heat

A substantial amount of energy is required for evaporation of water. Evaporation of water from the wet surfaces inside of leaves can dissipate a large amount of energy to balance the energy input from sunlight. Under unusual conditions, latent heat loss can exceed radiant heat gain, resulting in leaf temperatures below air temperature. Sensible heat exchange will then be positive, requiring additional heat loss by evaporation. Cotton is one of few plants that transpire enough water to substantially cool its leaves below air temperature. The large water loss cools the cotton leaves, in some cases by more then 5°C (Radin et al., 1994; Wise et al., 2004) and improves yield (Cornish et al., 1991), demonstrating that high leaf temperature limits yield in cotton.

Because latent heat loss is an important component of the energy balance of leaves, high temperature stress can be a consequence of drought, if plants do not have water available for transpirational cooling.

1.4. Conditions of High Temperature Stress

In light of the energy balance considerations, three conditions of high temperature stress can be described: (1) when air temperature is high, plants will tend to come to the high temperature by sensible heat transfer, (2) at the soil surface, where sunlight can cause the temperature to be substantially above air temperature (Campbell and Norman, 1998) and (3) in leaves where the substantial radiant heating by sunlight and the low heat capacity of leaves conspire to heat leaves very rapidly to as much as 15°C above air temperature. Using fine-wire thermocouples it has been shown that leaves with low transpiration rates, such as oak leaves, suffer frequent high temperature episodes (Singsaas and Sharkey, 1998; Hanson et al., 1999; Singsaas et al., 1999). However, most crops transpire more than the oak trees measured by Singsaas et al. (1999) and in

cotton, which normally grows in warm environments, stomatal conductance can be so high that leaf temperature is substantially below air temperature. In fact, it has been specifically shown for cotton that the greater the transpirational cooling, the greater the yield (Radin et al., 1994; Lu et al., 1997) proving that heat-stress-induced reductions of photosynthesis limit overall yield. Other studies have also linked reduced crop yields to moderate heat stress of leaves that reduces photosynthesis (Al-Khatib and Paulsen, 1990; Guilioni et al., 2003).

Some important effects of energy balance can be seen in the oak leaf temperatures shown in Figure 1. During the day, leaves at the top of the tree (black dots) can be over 10°C above air temperature because of the radiant heat load, while leaves at the bottom of the tree (light gray dots) are slightly below air temperature because of latent heat loss. At night, leaves at the top of the tree lose radiant heat to the sky and so are cooler than leaves at the bottom of the tree because leaves at the bottom of the tree exchange radiant heat with other leaves and with the ground, both of which are warmer than the sky.

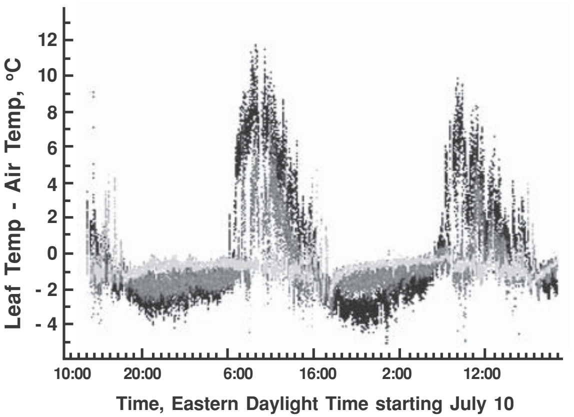

Figure 1. *Difference between leaf and air temperature for two days for three oak (Quercus rubra) leaves at the top (black dots) middle (dark gray) or bottom (light gray) of the canopy*

Leaves exposed to full sunlight will carry out most photosynthesis but also be subject to the rapid, high temperature spikes shown in Figure 1. We have started calling this heat flecks, analogous to (and sometimes simultaneous with) sunflecks (Pearcy et al., 1996). Because leaves are often the hottest parts of plants, high temperature stress is especially relevant to photosynthesis. Moreover, plant growth often increases with temperature to well above 30°C while photosynthesis of individual leaves typically has a temperature optimum of 30°C or less. At low temperature growth may be more directly

affected by temperature than photosynthesis. This situation can be summarized simplistically to growth may limit photosynthesis at low temperature but photosynthesis will limit growth at high temperature. Therefore, high temperature stress effects on photosynthesis are especially important.

2. EFFECTS OF HIGH TEMPERATURE STRESS

Biological systems can operate over a very wide range of temperature. Deep sea vent organisms live at >110°C (Blöchl et al., 1997) while other organisms survive < -40°C and many organisms are biologically active at <0°C. Biological systems rely on hydrophobic interactions and hydrogen bonding within and among the macromolecules of life. Hydrophobic interactions at room temperature result from the increase in entropy that occurs when hydrophilic and hydrophobic substances are mixed. Given the significant temperature dependence of entropy effects on Gibbs free energy ($\Delta G = \Delta H + T\Delta S$), the strength of hydrophobic interactions will be strongly temperature dependent. Membranes self assemble from amphipathic lipids as a result of hydrophobic interactions. Therefore, their properties will depend critically on temperature. Membrane components suited to one temperature will be inappropriate at other temperatures. Protein structure is determined by both hydrophobic effects and hydrogen bonding (as well as some covalent bonds) (Pace et al., 1996) and it too is very strongly affected by temperature. Changes in amino acid sequence needed to adjust proteins for activity at different temperatures can be subtle (Haney et al., 1999; Vieille and Zeikus, 2001; Muslin et al., 2002; Maeda et al., 2002). Both membranes and proteins need to be flexible in order to function in biological systems; this restricts the temperature range over which they can be biologically active (Haney et al., 1999). Thus, high temperature stress occurs when organisms experience temperature above that to which they are adapted and that adaptation depends strongly on the makeup of the proteins and membranes of an organism.

2.1. Membrane Properties

Membranes have to be strong enough to divide cells from their surroundings and into compartments, but also fluid enough to allow proteins to move. The properties of membranes can be adjusted to increase the temperature range tolerated by plants by changing the fatty acids in their membranes (Pearcy, 1978). Membrane lipids often have less unsaturated fatty acids when grown at high temperature and this was associated with tolerance of photosynthesis to high temperature stress (Raison et al., 1982). Later researchers, working with cyanobacteria, were unable to find a strong relationship between thylakoid glycerolipid unsaturation and heat tolerance of photosynthesis (Gombos et al., 1994). This is in contrast to very clear relationships between photosynthetic tolerance of low temperature and glycerolipid composition (Moon et al., 1995; Ariizumi et al., 2002). However, recently, heat tolerance has been shown to be increased

when *Arabidopsis* plants were modified to lack essentially all capability to make triply unsaturated thylakoid lipids (Murakami et al., 2000). Thus, there is clear evidence that thylakoid membranes can affect plant tolerance to high temperature, and that this can affect overall plant thermotolerance, but the effect may not be universal. The importance of membrane fluidity in high temperature stress remains an unsettled question.

2.2. Proteins and Heat Stress

Because of the temperature sensitivity of the forces responsible for protein folding (Pace et al., 1996), proteins are easily denatured by high temperature. Biological organisms have a suite of proteins that are made in response to high temperature that appear to be designed to prevent or reverse the effects of heat on protein denaturation. These are called heat shock proteins because they are made in abundance when organisms are subjected to potentially damaging high temperature. Many of these proteins are also made in response to other stresses, notably osmotic and oxidative stress. It is believed that these proteins help other proteins fold correctly or refold after damage by heat stress (Boston et al., 1996) although recent data has established unique roles for each of the families of heat shock proteins.

Heat shock proteins were discovered as proteins that were not expressed or not very prominent in assays of plants grown in moderate temperature but which became prominent when organisms were subjected to a heat shock (Lindquist, 1986). The hsps are divided into families based on their molecular weight. For example, most organisms have heat-inducible genes with a molecular weight between 100 and 104; these are called Hsp100. The different hsp families appear to have different functions. Some are constitutive, expressed at a somewhat higher level in response to heat stress, while others are nearly completely inducible, with no protein detectable in unstressed plants and lots of protein in stressed plants. The following is a quick tour of some of the important aspects of each class of heat shock proteins.

Hsp 100 - This class of hsp has been shown to be directly related to thermotolerance (Queitsch et al., 2000). Plants lacking Hsp101 can grow at normal temperatures and reproduce, but unlike wild type plants, a pretreatment at moderately high temperature does not induce thermotolerance. Hsp101 is produced by *Arabidopsis* seedlings and confers tolerance to heat stress (Hong and Vierling, 2000; Queitsch et al., 2000) but there is now evidence for an additional three genes that are not hsps that contribute to inducible thermotolerance as measured in hypocotyl elongation assays (Hong et al., 2003). Expression of heat shock proteins and heat shock factors (proteinaceous factors that stimulate expression of heat shock proteins as well as other heat tolerance genes) results in increased thermotolerance in many plants (Sun et al., 2002). Hsp101 is highly inducible and plants that have not experienced high temperature have only very low level, if any, Hsp101. The Hsp100 family has sequence similarities to caseinolytic protease B (*ClpB*) and is often referred to as *Hsp100/clpB* (Agarwal et al., 2002). These proteins have nucleotide binding mo-

tifs and appear to recruit other hsps to help repair damaged proteins. In yeast, Hsp104 can reverse the effect of high temperature on protein structure. Yeast Hsp104 acts with other hsps (Hsp40 and Hsp70) to undo damage to proteins caused by heat (Glover and Lindquist, 1998).

Hsp90 – In animals Hsp90 is best known as a component of a complex that helps fold the glucocoticoid receptor. In plants, Hsp90 participates in a similar multiprotein complex that also includes Hsp70 and a small acidic protein known as p23 (Pratt et al., 2001). Hsp90 complexes appear to be involved not only in protein folding, but also protein transport along microtubules inside the cell, especially transport of proteins into peroxisomes (Pratt et al., 2001). Hsp90 is essential for growth and is induced by low temperature (Krishna et al., 1995) in addition to being prominently expressed at high temperature. Hsp90 has been called a "capacitor" for genetic variation. In this view, Hsp90 can cover up developmental abnormalities, but when Hsp90 levels are reduced, these abnormalities get expressed and this, in essence, creates a burst of evolution in response to a stressful environment (Queitsch et al., 2002).

Hsp 70 – Like Hsp90, Hsp70 is required for plant growth and is essential for processes such as protein import into chloroplasts and mitochondria (Marshall et al., 1990). Hsp70 proteins appear to be involved in virus infectivity and perhaps in allowing viruses to move between cells of plants through plasmadesmata (Alzhanova et al., 2001).

Hsp60 – Rubisco folding requires a "rubisco binding protein" that appears to be a member of the Hsp60 family. As many as 14 Hsp60 protein chains will aggregate to make a functional complex to fold rubisco (Roy and Andrews, 2000). Hsp60s are essential for folding of many proteins in plants and it is presumed are needed for refolding when proteins are denatured by heat.

smhsp (mol wt 17 to 28 kDa) – The family of small heat shock proteins is much bigger in plants than other organisms such as fungi or animals (Vierling, 1991). It has been hypothesized that the rapid divergence of small heat shock proteins in plants into five or six families may have been an important step in the evolution of plants onto land required to allow plants to cope with the large temperature fluctuations that occur in air with its low heat capacity relative to water (Waters, 1995, 2003). The families of small heat shock proteins in plants are located in compartments, one in the chloroplast, one in the endoplasmic reticulum, and two families in the cytosol. It was recently shown that there are two small hsp families in the mitochondrion (Scharf et al., 2001). The plant small hsps appear to play a role in thermotolerance of a number of processes (Basha et al., 2004). Modulation of thermotolerance in carrot cell cultures has been reported by modulating the abundance of a small hsp (Malik et al., 1999). The chloroplast small heat shock proteins have been associated with increased thermotolerance (Wang and Luthe, 2003) and the mechanism has been suggested to be protection of photosystem II (Heckathorn et al., 1998,

2002). The small Hsps have a motif that is closely related to the α-crystallin protein in the lens of animal eyes. Another family of small proteins in *Arabidopsis* also contains α-crystallin domains and are called Acd proteins but their role in heat tolerance is unknown . α-crystallin undergoes a change when exposed to temperatures between 38 and 50°C that causes an increase in surface hydrophobicity (Das and Surewicz, 1995). It is not clear why the small hsps should be related to α-crystallin nor whether the increased hydrophobicity of α-crystallin is related to the chaperone activity of small hsps.

Many of the hsps are required during high temperature stress. Recent evidence indicates that there are heat shock factors with DNA binding domains that may be important components in the transduction pathway between high temperature stress and gene expression leading to accumulation of heat shock proteins (Nover et al., 2001). There appear to be two classes of heat shock transcription factors in *Arabidopsis* (Czarnecka-Verner et al., 2000).

In summary, heat stress may affect membrane function and protein folding. The relative importance of these two effects probably depends upon which plant process is being considered.

3. SPECIFIC PLANT PROCESSES AFFECTED BY HIGH TEMPERATURE

Heat stress can affect some plant processes more than others. Extreme temperature will affect many processes, but the most important effects are those that are first encountered as temperatures rise above the optimum for plant growth. Two plant processes that are particularly sensitive to heat stress are pollen development and photosynthesis. Other processes that appear to be inherently less sensitive to heat stress include respiration (Berry and Raison, 1981). Pollen development and fruit set are critical to making the parts of plant we most often harvest as a crop. The effect of heat stress on crop yield will depend upon the timing of the heat stress. If the stress is experienced during anthesis, substantial loss in fruit set and, ultimately, crop yield can occur. Photosynthesis, on the other hand, is also particularly sensitive to heat stress and heat stress at nearly any time during crop growth could have adverse consequences on yield through stress effects on photosynthesis (Guilioni et al., 2003). Because pollen development and photosynthesis are the two most high temperature-sensitive plant processes, the next sections will cover the effects of high temperature on these processes.

3.1. Pollen Development

Pollen development is particularly sensitive to high temperature stress. High temperature at anthesis can prevent fruit set in tomato (Peet et al., 1998; Sato et al., 2000) and

many other species (Porch and Jahn, 2001; Aloni et al., 2001; Pechan and Smykal, 2001; Kakani et al., 2002; Cross et al., 2003; Young et al., 2004).

To separate effects of heat on pollen and fruit development, Peet et al. (1998) used male fertile and male sterile tomatoes grown at daily mean temperatures of 25°C, 27°C, or 29°C. Male fertile tomatoes that were self-pollinated yielded about 10% of the fruit mass at 29°C that they did at 25°C. Make sterile plants pollinated with pollen from male fertile plants grown at 29°C yielded no fruit regardless of the temperature at which the receiving plants were grown. However, when pollinated with pollen developed at 25°C plants grown at 29°C yielded 73% of the amount of plants grown at 25°C. This shows clearly that microsporogenesis is very sensitive to temperature in tomato, more so than fruit development.

Prasad et al. (2002) grew common bean (*Phaseolus vulgaris* L.) at five temperature regimes between 28°C/18°C day/night and 40°C/30°C day/night. These plants exhibited substantial loss in pod set above 37°C/27°C day/night temperature and reductions in seed set at even lower temperature (Prasad et al., 2002). Pollen viability was even more sensitive and was more than 50% inhibited in the plants grown at 37°C/27°C day/night temperature. These effects were not sensitive to increased CO_2, unlike some effects of temperature on photosynthesis (Cowling and Sage, 1998). This is consistent with the effects being primarily on pollen viability and development of reproductive structures rather than on photosynthesis.

Pima cotton (*Gossypium barbadense* L.) grown at elevated CO_2 had greater yield at low to moderate temperature, but at 35°C, yield was reduced and there was no stimulation caused by growth in high CO_2 This indicates that the effects of high temperature was not mediated by a reduction in photosynthesis (Reddy et al., 1995).

In contrast to the lack of effect of CO_2 reported above, Aloni et al. (2001) found that bell pepper (*Capisicum annuum* L.) pollen from plants grown at elevated CO_2 had higher germination rates. This effect was ascribed to a higher carbohydrate status of the pollen and the authors suggested that high temperature can cause pollen to run out of reserves too soon, resulting in loss of viability. A heat-tolerant cowpea [*Vigna ungulata* (L) Walp] variety had greater yield in elevated CO_2 when grown at 33°C/30°C day/night but heat sensitive varieties did not become more tolerant as a result of growth in elevated CO_2 (Ahmed et al., 1993). In this experiment, only night temperature was varied and reproductive failure in the sensitive varieties resulted from increasing the night temperature from 20°C to 30°C while maintaining the day temperature at 33°C. Reproductive failure was associated with low levels of sugar in the peduncle. Thus, carbohydrates seem to be involved in pollen viability but the effect of high temperature on pollen viability and seed set is not directly a result of temperature on photosynthesis.

In rice, grain yield is reduced by increasing temperature and in this species, elevated CO_2 caused a *decrease* in yield at the same high temperature (Matsui et al.,

1997). The authors of this study concluded that stomatal closure resulted in less evaporative cooling in the canopy and so an increase in canopy temperature relative to a crop grown in today's level of CO_2 and at the same air temperature. Thus, energy balance considerations can cause elevated CO_2 to reduce grain yield. Because rice is a major crop and is grown in many environments near its high temperature limit, the effect of high temperature stress on reproductive yield is a pressing problem deserving of substantial effort, if major crop failures are to be avoided as global temperatures rise.

3.2. Seedling Establishment

Heat can limit plant growth at the seedling stage, because the temperature near the soil can be very high as a result of a boundary layer of air near the soil surface (Campbell and Norman, 1998). Soil temperature can exceed 50°C when the sun is bright. Metabolism in seedlings of many species can respond to heat through the induction of hsps (Vierling, 1991). Many of the studies of hsp-derived thermotolerance use assays of seedling establishment (Hong and Vierling, 2000; Queitsch et al., 2000; Hong et al., 2003), which is appropriate, even though hsps have effects well beyond their effect on this phase of the plant's development.

3.3. Photosynthesis

Even in the absence of any injury, photosynthesis of C_3 plants would be expected to decline as temperature increases because photorespiration increases with temperature faster than does photosynthesis (Schuster and Monson, 1990). However, it is also well known that heat directly damages the photosynthetic apparatus, with photosystem II often considered a key weak link (Santarius, 1975; Santarius and Müller, 1979; Berry and Björkman, 1980) but only above 45°C (Terzaghi et al., 1989; Thompson et al., 1989; Gombos et al., 1994; Çajánek et al., 1998). One effect of high temperature is destruction of the oxygen-evolving complex with the loss of a 33 kDa extrinsic protein and Mn^{2+} (Enami et al., 1994b). However, while reversible reductions in PSII-dependent electron transport can be seen at less than 40°C, irreversible effects and loss of the 33 kDa protein occur at >42°C (Yamane et al., 1998). Thus, damage to PSII cannot explain widely observed, heat-induced depression in photosynthesis seen at temperatures between 35°C and 40°C. This chapter will focus primarily, but not exclusively, on the effects of moderately high temperature (35°C to 40°C) on photosynthesis.

3.3.1. The Role of Stromal Proteins

There are some studies linking heat shock proteins and photosynthetic capacity (Heckathorn et al., 1998; Downs et al., 1999; Heckathorn et al., 2002; Barua et al., 2003). The chloroplast-localized small hsp appears to be correlated with temperature range of

plants. Its mode of action has been suggested to be protection of PSII. However, PSII damage does not occur under moderate high temperature stress.

Moderately high leaf temperature leads to deactivation of rubisco (Kobza and Edwards, 1987) confirming the findings of (1981b) that rubisco activity is reduced by heat. This deactivation can occur at temperatures that cause no harm to PSII (Feller et al., 1998) and has been proposed to be the primary constraint to photosynthesis at moderately high temperature (Crafts-Brandner and Salvucci, 2000). The deactivation is presumed to result from loss of activity of rubisco activase, which is very sensitive to denaturation by high temperature (Salvucci et al., 2001) or perhaps binding of activase to the thylakoid membrane (Rokka et al., 2001).

While rubisco deactivation is easily demonstrated at moderately high temperature, it is not clear whether that is the cause or a consequence of the reduction of photosynthesis. Generally, rubisco activation is modulated to match rubisco activity to the activity of the other components of photosynthesis (Sage, 1990). Schrader et al. (2004) found that NADP-dependent malate dehydrogenase activity, a proxy for stromal redox status (Scheibe, 1987; Scheibe and Stitt, 1988), was reduced to the same extent as rubisco activation by moderately high temperature. Two pieces of evidence suggest that the rubisco deactivation is not the primary effect of moderately high temperature. First, in genetically modified tobacco plants that lack rubisco activase and so have a low but constant activation of rubisco, moderately high temperature inhibits photosynthesis to the same degree as the wild type plant. However, moderately high temperature stress of photosynthesis in the activase lacking plant is irreversible, while the inhibition in plants in which rubisco activation falls is rapidly reversed when the leaves are returned to non-stressful temperature (Sharkey et al., 2001a). Second, by analyzing the response of photosynthesis to CO_2 in leaves of cotton at a variety of temperature, it was shown that the functional limitation was electron transport and not rubisco activity or activation (Wise et al., 2004). By applying the Farquhar model of photosynthesis (Farquhar et al., 1980) and temperature responses of the model parameters (Bernacchi et al., 2001, 2002, 2003) it was shown that rubisco activity could not explain the CO_2-response of leaves at temperatures between 30°C and 40°C (Wise et al., 2004).

3.3.2. The Role of Membranes

Rising temperatures increase the fluidity of the thylakoid membranes (Raison et al., 1982), and in some cases cause the formation of non-bilayer structures (Gounaris et al., 1983). High temperatures also uncouple electron transport from photophosphorylation (Emmett and Walker, 1969, 1973), presumably due to an increase in ion permeability of the thylakoid membranes (Weis, 1981a; Havaux et al., 1996; Schrader et al., 2004). However, this increased permeability and uncoupling due to heat stress is not associated with a decline in the energy gradient across the thylakoids as measured by 9AA fluorescence (Bukhov et al., 1999), but is associated with an increased qN (Feller et al., 1998; Law and Crafts-Brandner, 1999; Crafts-Brandner and Salvucci, 2002) which is indicative

of an increased pH gradient (Horton et al., 1996). Further, ATP levels, which are dependent on an energized thylakoid membrane, were higher in isolated chloroplasts (Weis, 1981a) and did not change in whole leaves (Schrader et al., 2004) under heat stress.

3.3.3. The Role of Membrane-Bound Reactions

Early studies of high temperature stress on photosynthesis noted a decline in oxygen evolution in isolated chloroplasts (Holt and French, 1946). Katoh and San Pietro (1967) ascribed this heat effect on O_2 evolution to the water splitting complex of PSII, showing that NADP photoreduction could be restored using ascorbate as an artificial electron donor that substituted for the water splitting complex. Later, the heat inactivation of O_2 evolution was associated with the release of Mn from PSII (Cheniae and Martin, 1970; Kimimura and Katoh, 1972; Wydrzynski and Sauer, 1980) and the release of the 18, 24, and 33 kDa proteins from the thylakoid membranes which are associated with the water splitting complex on the luminal surface of PSII (Figure 2) (Yamamoto and Nishimura, 1983; Nash et al., 1985).

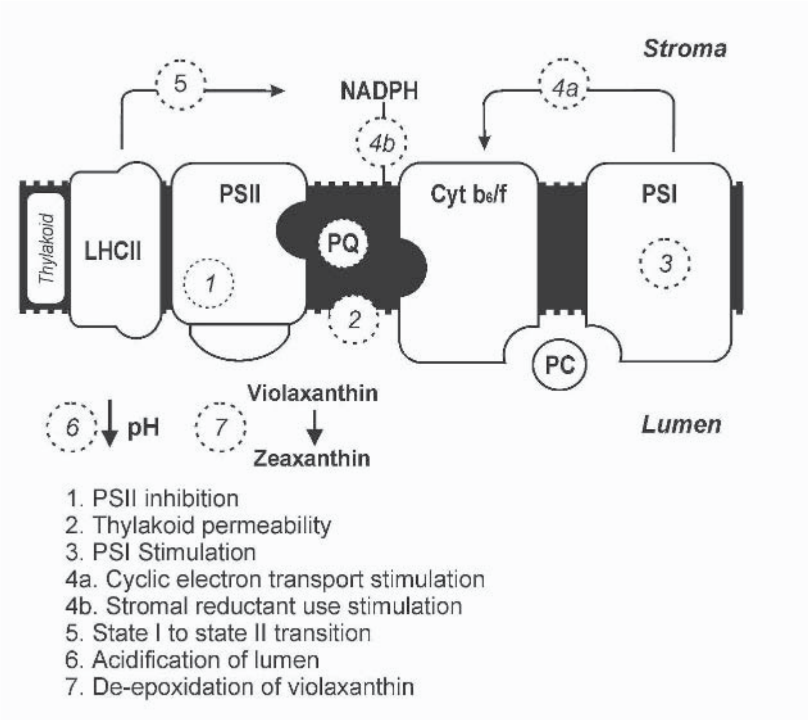

Figure 2. *Photosynthetic electron transport and processes that can be affected by high temperature stress (Light arrows indicate electron transport paths, heavy arrows indicate changes that can occur in response to heat)*

Manganese is an essential element of the water splitting complex of PSII, which oxidizes water and transfers the resulting electrons to P680. Therefore, the observed loss of manganese during inhibition of O_2 evolution suggests that heat is destroying the Mn complex. However, immobilization of the 33 kDa protein of PSII stabilized the water splitting complex and prevented the loss of Mn during heat stress (Enami et al., 1994b). Further, these studies showed that O_2 evolution could be completely inhibited by heat stress while 80% of Mn remained bound to PSII, suggesting the requirement of both Mn and the 33 kDa protein for O_2 evolution. The 33 kDa protein is a thermally stable protein, although its secondary structure is affected by heat stress, which dissociates from PSII upon heating (Enami et al., 1994a; Lydakis-Simantiris et al., 1999). Upon cooling, a portion of the 33 kDa proteins reassociate with PSII. This accounts for the observed partial recovery of O_2 evolution after heat stress. Pueyo et al. (2002) also demonstrated that the thermal stability of O_2 evolution in spinach could be enhanced by substitution of the native 33 kDa protein with a homologue from the thermophilic cyanobacterium *Phormidium laminosum* that has a more thermally stable protein secondary structure. This suggests that the thermal stability of the secondary structure of the 33 kDa protein is important for binding with PSII under heat stress conditions.

Although inhibition of O_2 evolution was the earliest noted effect of heat on PSII, high temperature also blocks the reaction center of PSII and causes a dissociation of the light harvesting pigment from the reaction center (Schreiber and Armond, 1978). While electron transport could be restored in heat damaged PSII using electron donors, other studies noted that full recovery of electron transport could not be obtained after heating with electron donors that supplied electrons before plastoquinone, suggesting further damage to PSII apart from the dissociation of the water splitting complex (Yamashita and Butler, 1968). This thermal blocking of the reaction center of PSII has been attributed to either a slowed rate of electron flow from Q_A- to Q_B or a back flow of electrons from Q_B- to Q_A using triazine resistant plants (Ducruet and Lemoine, 1985; Ducruet and Ort, 1988; Havaux, 1989; Ducruet, 1999) and in *Amaranthus* chloroplasts (Bukhov et al., 1990). Further, Egorova et al. (2003) showed that the reduction of plastoquinone by stromal reductants during heat stress caused a back pressure of electrons on PSII thus causing a reduction of Q_A by Q_B-. This strongly suggests that the thermal blocking of PSII noted first by Schreiber and Armond (1978) is due to the reduction of plastoquinone from stromal sources.

The dissociation of the light harvesting pigment from the reaction center during heat stress has been attributed to the disconnection of the LHCII complexes from the functional core of PSII. Several freeze fracture studies have shown a decrease in particle size located in the thylakoid membranes after heat stress, indicating a physical separation of the LHCII complexes from the core of PSII (Armond et al., 1980; Gounaris et al., 1983, 1984). This dissociation is further linked to either a state I to state II transition in leaves heated to 42°C or to the formation of aggregates of LHCII complexes in the thyalkoid membranes in leaves heated to 45°C and above (Pastenes and Horton,

1996a; Gounaris et al., 1984; Sundby et al., 1986; Joshi et al., 1995). Changes in the structure of the thylakoid membrane can be seen in electron micrographs (Armond et al., 1980; Gounaris et al., 1984) of plants treated for short periods of time at moderately high temperature.

Finally, a pronounced stimulation of PSI activity by heat stress has been reported a number of times (Pastenes and Horton, 1996a; Bukhov et al., 1999; Schrader et al., 2004). The stimulation of PSI activity comes at the expense of the redox status of the chloroplast (Bukhov et al., 2001; Egorova and Bukhov, 2002; Schrader et al., 2004). PSI-mediated cyclic electron flow (which would generate the transthylakoid energy gradient needed to regulate PSII (Demmig-Adams and Adams, III, 1992) could be a mechanism for limiting PSII activity and so limit the production of AOS (Heber and Walker, 1992; Heber, 2002).

3.3.4. Cyclic Electron Flow around Photosystem I

Contrary to the detrimental effects of heat on PSII and thylakoid membrane permeability, it was noted early that heat stimulated PSI activity (Stidham et al., 1982; Monson et al., 1982), and that this stimulation coincided with the inhibition of PSII activity (Thomas et al., 1986a). Thomas et al. (1986b) demonstrated in isolated chloroplasts that PSI stimulation was not due solely to uncoupling of photophosphorylation and that granal stacking had no influence on heat stimulation of PSI, but that PSI reaction centers turned over more rapidly under heat. They also suggested that a new electron acceptor site was exposed on cytochrome b_6/f, indicating that cyclic electron transport may be important in the heat stimulation of PSI.

Heating appears to specifically engage cyclic electron flow around PSI (Havaux, 1996; Bukhov et al., 1999, 2000). The dark rereduction of PSI was found to undergo "spectacular acceleration" with the half-life of $P700^+$ falling from over 500 ms to less than 50 ms between 34°C and 40°C (Havaux, 1996). Havaux noted that the rise in PSI activity with heat stress was catalyzed by electron flow from stromal reductants through plastoquinone, which was further confirmed by several other studies (Bukhov et al., 1999, 2000, 2001). A flow of electrons from the stroma to the plastoquinone pool in the dark at 36°C was reported by Yamane et al. (2000). In these studies a specific protein was hypothesized that might catalyze electron donation to plastoquinone and that would only be active above 35°C. Yamane et al. (2000) found little effect of antimycin A, the inhibitor of ferredoxin plastoquinone reductase-type cyclic electron flow. They also saw only minor inhibition of the high temperature electron flow to plastoquinone by feeding inhibitors of plastidial NAD(P)H dehydrogenase complex (Ndh1), the protein which catalyzes the other known cyclic electron transport path. The Ndh-dependent cyclic electron transport pathway was found to be a high capacity pathway in low oxygen or when mitochondrial and chlororespiratory metabolism was poisoned (Joët et al., 2002). Sazanov et al. (1998) found that the plastoquinone pool was still being

reduced after heat stress in the light in *ndh* mutants, suggesting alternate electron routes. On the other hand, Bukhov et al. (2001) found that the heat-stimulated cyclic electron transport was sensitive to rotenone and so concluded that NADPH is the primary stromal reductant involved. Thus, increased cyclic electron transport may lead to a decline in the NADPH levels inside the stroma as more electrons are diverted back into the electron transport chain.

Several authors have found a decline in NADP-MDH activity, which is dependent on the stromal redox status and the NADPH/NADP$^+$ ratio, in heat treated bean and cotton leaves as low as 35°C (Pastenes and Horton, 1996b; Schrader et al., 2004). However, Weis (1981b) found no decline in NADP-MDH activity at 38°C in isolated chloroplasts, and Sharkey et al. (2001a) found no decline in NADP-MDH activity in WT tobacco leaves and an increase in activity in rubisco-activase-deficient transgenic tobacco leaves at 40°C.

During a 39°C heat pulse, we demonstrated a stimulation of PSI with little concurrent effect on PSII (Schrader et al., 2004) thus confirming the work of others that cyclic electron transport is dramatically accelerated during heat stress. The stimulation of PSI happens at lower temperature than the inhibition of PSII usually associated with heat stress (Terzaghi et al., 1989; Thompson et al., 1989; Gombos et al., 1994; Cajánek et al., 1998). The finding that lowered redox status can lead to significant stimulation of PSI-mediated cyclic electron transport (Joët et al., 2002) raises a question.

One of the mechanisms involved in stimulating cyclic electron flow is phosphorylation of light harvesting chlorophyll complex of PSII (LHCII). Phosphorylated LHCII moves from the appressed thylakoid regions, where PSII is located, to the unappressed regions, where PSI is located (Chow et al., 1991). The phosphorylated LHCII becomes energetically disconnected from PSII core complex (slowing its turnover rate) and energetically coupled to a PSI core (increasing its turnover rate) and this process requires a specific polypeptide within PSI (Lunde et al., 2000). This is the well known state transition (Allen, 1992) and is known to accompany an increase in cyclic electron flow around PSI. However, heat (which also stimulates PSI-mediated cyclic flow) has been reported to stimulate *de*phosphorylation of a number of PSII core proteins namely D1, D2 and CP43 (Rokka et al., 2000; Vener et al., 2001). Therefore, the regulation of phosphorylation of LHCII may well be quite different from the regulation of phosphorylation of the PSII core proteins (Harrison and Allen, 1991; Pursiheimo et al., 2003). The regulation of phosphorylation of thylakoid proteins interacts with redox status (Vener et al., 1995). Likewise, dephosphorylation of LHCII appears to be catalyzed by a different phosphatase than dephosphorylation of other thylakoid-associated proteins (Hammer et al., 1997; Vener et al., 1999). There are several thylakoid associated kinases (TAKs) (Snyders and Kohorn, 1999, 2001) but also another kinase that appears unrelated to TAKs that is necessary for state transitions and phosphorylation of LHCII (Depège et al., 2003).

Thus, there may be two different regulatory systems that control thylakoid protein phosphorylation/dephosphorylation, one that controls phosphorylation /de-

phosphorylation of LHCII and state transitions and a second system that controls phosphorylation/dephosphorylation of the other thylakoid proteins. A large number of thylakoid proteins undergo reversible phosphorylation (Hansson and Vener, 2003) and heat stress is one of the most effective ways of modulating the phosphorylation status of many of them (Vener et al., 2001). For some thylakoid proteins whose phosphorylation status varies, the function of the protein is not known (Carlberg et al., 2003; Hansson and Vener, 2003).

One of the processes in which phosphorylation plays a role is repair of damaged D1 proteins. PSII complexes with a damaged D1 migrate from the stacked to unstacked thylakoids for repair, but the D1 has to be dephosphorylated before the repair can occur (Rintamäki et al., 1996). Migration and dephosphorylation of D1 are reduced at low temperature, which could explain why unsaturation of thylakoid lipids, which is deleterious at high temperature (Kunst et al., 1989; Murakami et al., 2000), is advantageous at low temperature (Vijayan and Browse, 2002). When the membrane is too rigid, PSII particles cannot migrate to the unstacked thylakoids as needed for repair.

3.3.5. Thermoprotection

Heat stress clearly causes a decline in the carbon assimilation of photosynthesis, but it is not certain what the primary cause of this observed decline is. Three components of the photosynthetic machinery have been suggested to be the primary heat labile component: PSII, lipid permeability, and rubisco activase. The other observed phenomenon during heat stress can be seen as protective mechanisms designed to protect either PSII or the lipid membrane. No protective mechanisms aside from a modified enzyme have been shown for rubisco activase. Further, several mechanisms have been shown to increase thermal stability in photosynthesis that are not directly involved with the conversion of light energy or the fixation of CO_2.

Much of the protection of PSII and the lipid membranes seems to center on cyclic electron transport. The induction of cyclic electron transport during heat stress would explain many observed phenomenon including the stimulation of PSI while PSII is inhibited, the reduction of the intersystem electron transport chain by stromal reductants and the subsequent blocking of the PSII reaction center, the state I to state II transition, the acidification of the lumen while the lipid membrane becomes progressively leakier, and the subsequent increase nonphotochemical quenching, qN. The protection provided by cyclic electron transport resides in the formation of the ionic gradient across the thylakoid membrane and nonphotochemical quenching (Horton et al., 1996). It is beyond the scope of this review to discuss the mechanisms of nonphotochemical quenching, but a review of its importance with heat stress is discussed. Interestingly, if the formation of a high pH gradient across the thylakoid membranes is protective during heat stress, then the deactivation of rubisco may be an adaptive response rather than maladaptive.

Nonphotochemical quenching of PSII chlorophyll fluorescence is attributed to three components: energy dependent quenching, qE, photoinhibitory quenching, qI, and state transition quenching, qT. The effects of qI are not discussed in this review. Energy dependent quenching, qE, results from the acidification of the lumen and is closely associated with PSII and LHCII activities, the deepoxidation of violaxanthin to zeaxanthin in the xanthophyll cycle, and in photoprotection. It is assumed that a large portion of heat related qN is due to qE because of the increased pH gradient and induction of cyclic electron transport during heat stress. Although, qT may play a significant role as it was noted earlier that mild heat stress causes a state I to state II transition.

An important protective mechanism for PSII is the de-epoxidation of violaxanthin to zeaxanthin. Havaux and Tardy (1996) noted an increase in zeaxanthin during a 2 hr heat stress at 35°C in potato leaves and a concurrent increase in the thermal stability of PSII, indicating that this protection is also important at elevated temperatures. Further, Havaux et al. (1996) noted a stabilization of the thylakoid membranes to heat stress by increasing the amount of zeaxanthin in leaves with ascorbate.

A high ion gradient across the thylakoid membranes may also increase ionic interactions at the lipid headgroups and thus stabilize the structure of the thylakoids. Several authors noted a protective effect of light, which presumably forms a pH gradient across the thylakoid membranes, on photosynthesis. Weis (1981a) found that the 518 nm electrochromic shift decay rate was less when chloroplasts were incubated in the light rather than the dark. Havaux et al. (1991) found that PSII fluorescence was stabilized when leaves where heated in the presence of light rather than the dark. The induction of cyclic electron transport and the deactivation of rubisco may be adaptive responses to high temperatures rather than maladaptive responses.

The dichotomy of observations showing an increased energy gradient across the thylakoid membranes while the permeability of the thylakoids increase during heat stress may be explained by a reduced ATP and NADPH requirement of the Calvin cycle because of the deactivation of rubisco and/or an increase in cyclic electron transport through PSI. An increased energy gradient and decreased lumen pH serve as protective mechanisms for PSII and the thylakoid membranes. The qE quenching of qN is well understood (Horton et al., 1996), and as mentioned earlier, it is also known that qN increases with temperature.

Many authors have noted interaction effects of various variables with heat stress, including thermoprotection from isoprene, solutes, and CO_2. Isoprene (C_5H_8, 2-methyl 1,3-butadiene) is produced in large quantities by plants and has been shown to increase the thermal tolerance of photosynthesis (Sharkey and Yeh, 2001). By removing endogenous isoprene using a nitrogen atmosphere and comparing this to leaves producing isoprene or adding exogenous isoprene to leaves, studies have found a shift to higher temperatures in leaves before heat damage occurs (Sharkey and Singsaas, 1995; Singsaas et al., 1997). Further, fosmidomycin-fed leaves showed greater tolerance

when fed exogenous isoprene (Sharkey et al., 2001b). Fosmidomycin inhibits the production of isoprene without interfering with photosynthesis. However, Logan and Monson (1999) did not find an enhanced thermal tolerance from exogenous isoprene on leaf discs. This led to the hypothesis that isoprene protected against heatflecks or short, high temperature events that leaves frequently encounter (Singsaas and Sharkey, 1998; Sharkey et al., 2001b). The exact mechanism of thermal tolerance due to isoprene is unknown, but it has been proposed that isoprene embeds in the thylakoid membrane and increases hydrophobic interactions (Sharkey and Singsaas, 1995; Sharkey and Yeh, 2001). The increase in hydrophobic interactions may have one or both of two effects. The first would be to stabilize the thylakoid membrane and decrease ion leakage or inhibit non-bilayer formation. The second may be to increase lipid protein interactions and thus stabilize proteins associated with the electron transport chain. If the increase in hydrophobic interactions in the thylakoid membrane is the reason for the increased thermal tolerance of photosynthesis, then other mechanisms that increase these hydrophobic interactions should also increase the thermal tolerance of photosynthesis, and this has been confirmed. Alkenes similar to isoprene in structure increased the photosynthetic thermal tolerance although alkanes did not (Sharkey et al., 2001b).

Plants grown at high temperature have a reduced degree of unsaturation in their membrane lipids (Pearcy, 1978). Mutant *Arabidopsis* plants, *fad7fad8* (Hugly et al., 1989; Kunst et al., 1989), and transgenic tobacco plants, with a silenced FAD7 gene, have increased lipid saturation show increased growth and photosynthetic thermal tolerance (Murakami et al., 2000). Thermal tolerance was also increased in chloroplasts and thylakoids when their lipids were chemically hydrogenated (Quinn et al., 1989).

Sugars and other solutes that do not interact with the lipid phase of thylakoid membranes have also been shown to provide increased thermal tolerance to thylakoid membranes and PSII (Feldman, 1962; Molotkovsky and Zhestkova, 1965; Molotkovsky, 1968; Santarius, 1973; Williams et al., 1992). Glycinebetaine deficient maize and transgenic Arabidopsis and rice showed decreased tolerance to heat stress (Yang et al., 1996; Alia et al., 1998; Kishitani et al., 2000). Glycinebetaine stabilizes PSII against thermal inactivation possibly by stabilizing the water splitting complex (Gorham, 1995; Allakhverdiev et al., 1996, 2003; Klimov et al., 2003). Digalactosyl diacylglycerol (Yang et al., 2002a, 2002b) and SQDG have also been shown to enhance thermal tolerance. Plants grown under enriched CO_2 atmospheres have shown a greater thermal tolerance in PSII chlorophyll fluorescence (Faria et al., 1996; Huxman et al., 1998; Taub et al., 2000; Hamerlynck et al., 2000). Bicarbonate has also been shown to interact with PSII and enhance thermal tolerance (Allakhverdiev et al., 1997; Klimov et al., 1997a, 1997b; Hulsebosch et al., 1998; Yruela et al., 1998; van Rensen et al., 1999; Klimov and Baranov, 2001; Klimov et al., 2003).

3.4. Translocation

At high temperature (above 35°C) transport of sugars out of leaves appears to be inhibited (Jiao and Grodzinski, 1996; Leonardos et al., 1996). Sugars accumulate in leaves held at high temperature showing that the translocation can be inhibited more than photosynthesis, especially in low oxygen (to prevent the photoreespiration induced reduction of photosynthesis). This is evidence that some aspect of translocation, possibly phloem loading, is inhibited by high temperature.

4. STRATEGIES FOR IMPROVING HEAT TOLERANCE

Improving heat tolerance has been attempted by biotechnological methods. One method is control of the composition of membranes. For example, Murakami et al. (2000) engineered *Arabidopisis* plants to prevent them from making trienoic fatty acids in their chloroplasts. This significantly improved the ability of these plants to survive heat stress.

A more common method for improving heat stress is engineering constitutive- or over-expression of Hsps. Hsp 100 family members and the smhsps have both been implicated in thermotolerance of plants (Queitsch et al., 2000; Heckathorn et al., 2002; Wang and Luthe, 2003).

Perhaps more promise lies in overexpressing heat shock factors (Prandl et al., 1998). These are genes that control the expression of other genes and so changing the expression of one heat shock factor will affect the expression of many genes that through evolution, have come to be controlled by the same control. Heat shock factors may not always work through transcriptional activation (Czarnecka-Verner et al., 2000) but some clearly show binding to promoters and other evidence of being transcription factors (Prandl et al., 1998; Zhang et al., 2003; Lohmann et al., 2004). In a recent report. a heat shock factor was shown to improve (Izu et al., 2004) the quality of male germ cells, one of the most temperature sensitive stages of the plant life cycle as it is in other organisms.

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CHAPTER 5

FREEZING STRESS: SYSTEMS BIOLOGY TO STUDY COLD TOLERANCE

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1. INTRODUCTION

It is generally acknowledged that acclimation to abiotic stress, particularly tolerance to sub-zero temperatures, is extremely complex. It is a dynamic process regulated primarily by temperature and days to weeks are required to achieve maximum cold hardiness. The rate and extent of cold acclimation while primarily influenced by temperature is also moderated by factors such as light intensity, day length, cultural practices, and other abiotic stresses such as drought and salinity.

In nature, cold acclimation is initiated by a combination of the shortening of the photoperiod which results in growth cessation and temperatures less than 10 °C. Some species are more sensitive to low temperature induction than others. For example winter rye (*Secale cereale*) increased in freezing tolerance when exposed to 10 °C, whereas winter wheat (*Triticum aestivium)* required 7 °C to initiate cold acclimation. In contrast, spring wheat did not increase in freezing tolerance until exposed to 4 °C. Thus, there appears to be differences in response to low temperature signaling. Exposure to other abiotic stresses (such as drought, UVB, wind, etc.) in the field at warm temperatures can also increase freezing. For example, seedlings of winter rye grown in the field at 25 °C can tolerate -9 °C, however, seedlings grown in a glass house at 25 °C

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only tolerate -3 °C (Gusta unpublished results). When acclimation to one stress results in increased tolerance to other stresses it is referred to as cross adaptation. However, the plant will not achieve the same level of freezing tolerance as attained by low temperature exposure. This suggests that some genes are specifically controlled by low temperature. The site of cold perception is not known, but is speculated to be due to a slight relaxation in the turgor of the plasma membrane (Sangwan et al., 2002), which results in changes in the intracellular pool of calcium. Not all tissues in a plant acclimate at the same rate and not all tissues achieve the same level of freezing tolerance. For example, the crowns of winter cereals are more freezing tolerant than the leaves while roots possess only a few degrees of freezing tolerance (Chen et al., 1983). Legg et al. (1983) observed that all tillers on winter cereals did not have the same LT_{50}. The point we are trying to establish here is although all tissues in a plant are genetically identical, morphologically and anatomically they are very different. These differences may impact the freezing tolerance that results from the upregulation of stress-associated genes.

Under natural conditions plants germinate, grow and mature under a constant state of environmental fluxes. Daily temperature variation can be as much as 10-15° C, however, it is very rare that a large temperature change of 20°C to 2°C occurs over a matter of a few minutes, especially in the rhizosphere. Due to the buffering action of the soil, changes in soil temperature and water availability are stable. In contrast, the roots of plants grown in pots are subjected to wide variations in temperature and water conditions, which do not reflect natural conditions. Depending upon the species and cultivar, plants grown in pots eventually recover after a period of days. Plants are exposed to large temperature variations during natural acclimation in the fall. For example, night temperatures may be lower than 8 °C, which triggers the upregulation of cold associated genes, but actual leaf temperatures during the day may readily exceed 20 °C, which promotes loss of freezing tolerance. Therefore, in the fall, the plant is exposed to a series of mixed signals. While the upregulation of cold associated genes are important for induction and maintenance of cold acclimation, equally important is the prevention in loss of freezing tolerance at non-acclimating temperatures. For example in nature, spring cultivars of *Brassica napus* rarely cold acclimates to -10 °C, however the same cultivar tolerates -19 °C when cold acclimated in controlled environment chambers. (Schilling, unpublished). Similarly non-acclimated winter cereals tolerate lower temperatures when hardened in a controlled environment chamber as compared to natural conditions (Gusta et al., 2001). Thus, the genes for freezing tolerance may be present in a genotype, however, the upregulation of the cold associated genes may not be optimized and therefore the regulation of cold-induced gene expression may be as important as the presence of any specific genes that directly confer cold tolerance. Another limitation to artificial acclimation is light intensity and quality. For example, very cold hardy winter wheat seedlings have a prostrate growth habit in the field, whereas plants in pots in controlled environment chambers subjected to cold acclimating temperature tend to have an erect growth habit. Photoinhibition can readily be induced in plants grown at constant low temperatures under high light conditions.

Whereas, in the field, symptoms of photoinhibition may only be apparent in the field prior to freeze occurring under full sunlight or when day temperatures approach 0 °C. At this time, the plants have reached their full cold hardiness potential and have also acclimated to high light and low temperature conditions (Strand and Öquist 1985). However, if plants undergo photoinhibition during the initial stages of hardening, the full hardiness potential will not be achieved because adequate photosynthates are not produced to drive the metabolism necessary for cold acclimation to occur. Thus, controlled environment chambers may produce an unrealistic picture of the process of cold acclimation. Cold acclimation is a multigene trait and it is very difficult to ascertain which genes are causal and which are induced by unrealistic, artificial conditions. The experimenter may argue since changes in nature are so slow and subtle it is difficult or impossible to measure changes in gene expression associated with cold acclimation, but the genes and proteins isolated from plants treated in this manner may represent a shock versus a stress response. This may or may not be true, but if many genes are upregulated that are only associated with a shock and not acclimation, how will the causal genes be discovered?

Artificial cold acclimating conditions can be a major limitation in identifying genes or proteins causally associated with freezing tolerance. Sudden changes in temperature can put the plant in a state of cold shock and the genes upregulated under these conditions may be more closely related to drought and chilling injury. For example, hardy temperate plants such as winter cereals of canola when transferred from a 20 °C to 4 °C display symptoms of wilting for the first 24 to 48 hours of transfer. Such dramatic changes in temperature rarely occur in nature and are not characteristic of the natural environmental signals. During natural cold acclimation which requires days to weeks, membranes become less saturated (Yoshida 1984), metabolism adjusts for low temperature growth (Levitt 1980) and photoinhibition is compensated for (Strand and Öquist 1985). In addition, pots in controlled environment chambers cool rapidly in contrast to the large mass of the earth's surface. Therefore, the roots undergo a shock that alters their hydraulic conductivity.

Therefore, some of the changes that occur when plants are exposed to rapid changes in temperature (20 °C to 4 °C within minutes) stress may not be related to cold acclimation. For example, Hammond et al. (2003) in a study on phosphate starvation reported the expression of 60 genes was transiently up-regulated four hours after withdrawing P. Several of these genes are involved in cell rescue and defense. These authors concluded that many of these genes are ubiquitous "shock" response genes up-regulated that have been shown to be upregulated by various pathogens and environmental perturbations (Desikan et al., 2001, Kreps et al., 2002). The following is a list of shock associated proteins; chitinases, peroxidases, and PR-1 like protein (Mackerness et al., 2001); cytochrome P450, a C2H2-type zinc finger protein and a blue copper-binding protein (Desikan et al., 2001) and proteins associated with reactive oxygen species (Gonzalez-Meler et al., 2001). Following the initial shock the genes up-regulated may be more closely related to the stress of interest. This lull in differential gene

expression will depend on the severity of the initial shock, the plasticity of the genotype and previous environmental perturbations.

2. SYSTEMS BIOLOGY

Systems biology, which presently encompasses transcriptomics, proteomics, and metabolomics, is a branch of biology whose objective is to discover and elucidate biological properties that emerge due to interactions of systems elements (Ideker et al., 2001; Kitano 2002). These new tools, based on high throughput computers, robotics and new tandem mass spectrometer chemistry have resulted in an explosion of information unparalleled in the history of biology. This information explosion presents new challenges for gathering and configuring data into interactive models. Thus, the development of predictive models will necessitate strong interactions with other disciplines such as mathematics, computer sciences, engineering, organic and biochemistry, evolutionary theories and bioinformatics. Perhaps the most important discipline for the success of system biology to add new and meaningful data will be supplied by the whole plant biologist who has a thorough knowledge of the growth and development of plants under natural environments. In studying abiotic stresses such as freezing, cooling and heating rates must be realistic, light intensities must approach sunlight, water potential changes should be similar to natural changes, and growth should not be restricted by the soil mass, etc. What appears to be forgotten is that many abiotic stresses occur over days to weeks and not in minutes. If experiments do not duplicate or approach natural conditions it does not make sense to add to the literature erroneous facts that confuse and retard, rather than advance, scientific truths. Controlled environment studies must be cross-referenced to field studies to obtain meaningful data.

The following sections on transcriptomics, proteomics and metabolomics discuss some recent research on abiotic stress with primary emphasis on cold acclimation. Due to the limitations of all these methods there will be a need for parallel analyses of transcripts, protein and metabolic profiles. There will then be a need for comprehensive identification of protein-coding genes, elucidation of protein structures and identification of protein functions, protein-protein interactions, localization of proteins, knowledge of signal transduction cascades to understand cellular dynamics and differentiation. Since cold acclimations is such a dynamic and complex multi-gene trait it will only be possible to identify candidate genes as system biology evolves. In all of these studies morphological and anatomical differences in plant tissues will need to be examined in order to determine their role in ameliorating stresses. Perhaps then it will be possible to make significant advances in understanding how plants adapt and tolerate abiotic stress.

3. MICROARRAY ANALYSIS

Microarray analysis is a method of global gene analysis that does not necessarily test theories or models but provides an avenue to explore the expression of thousands of

genes at the transcriptional level (Schena et al., 1995). Genomic sequencing projects have provided genetic information for entire genomes , however, the expression or function of many of these genes during a physiological process has yet to be determined. The ability to quantify and compare gene expression on a global scale is a powerful analytic tool. Microarray analysis makes it possible to potentially monitor and quantitate transcript levels of an entire genome in any biological state (eg. Response to pathogens, abiotic stress, developmental stage, etc.) (Brown and Botstein 1999; Duggan et al 1999). The information obtained from this approach can be used to elucidate biochemical and regulatory pathways. Candidate genes can be selected based on environmental condition, tissue, or physiological response and promoter regions determined for candidate genes using genomic DNA.

However, microarray analysis has some limitations. For example, this process can be limited by the diversity of biological samples used to generate the complimentary DNA (cDNA) libraries. The library developed may be limited by developmental stage, tissue type, or growth condition; therefore, changes in gene expression are restricted to the cDNA or expressed sequence tags (ESTs) isolated. Difficulty may arise in sampling a particular cDNA library to sufficiently identify low abundance transcripts and the use of genomic DNA over cDNA may increase the amount of hybridization with pseudogenes. The utility of this approach is hampered by the number of genes that exist in the database to which no known function has been assigned. Knowledge of the complete genome of the organism being studied would be ideal to understand gene expression such as *Arabidopsis* to reduce the number of unknown genes as well as isolate promoter regions.

Microarray analysis has proven to be a powerful tool to study gene expression and understanding the multiple response pathways that influence plant growth and development. Prior studies generally investigated a single gene product during stress and most often did not define the role of the stress-related gene stressing conferring tolerance to the stress. Microarray analysis has broadened the analysis of gene expression in response to a stress determining large scale changes in transcript profiles associated with stress and has confirmed the results of the single gene studies (Seki et al., 2001; Heath et al., 2002). Microarray analysis has been used to explore the role of individual genes and groups of genes expressed in plant tissues at various growth stages and under different environmental stress conditions (Fernandes et al., 2002). Fernandes et al. (2002) conducted a comparative analysis of maize gene expression to test the hypothesis that different structures express discrete sets of genes. The EST libraries were developed from corn leaf primordia, 10-14 day old endosperm, 1-2 cm immature ear, 4 day old root tissue, 1 mm tassel, greater than 2 cm tassel, mixed tassel stages, mixed embryo stages, and mixed adult organs. As a result, each tissue and development stage sampled in maize appeared to have a distinct set of moderate to highly expressed genes from specific gene families. The use of microarray analysis is not limited to the species the cDNA or ESTs were isolated from. Similar gene expression results can be obtained from closely related species. Girke et al. (2000) developed

microarrays that represented *Arabidopsis* seed expressed genes. The authors used the *Arabidopsis* array to profile the transcriptone of oilseed rape (*Brassica napus*) seeds and found expression patterns correlated well between the two species. Most seed specific probes identified in *Arabidopsis* provided seed specific signals in *B. napus*, however, the correlation coefficients for *B. napus* were slightly lower than those from *Arabidopsis*. These studies mentioned applied microarray analysis to isolate sets of genes expressed across tissues and between species. However, the use of microarray analysis can be expanded to study gene expression profiles of different tissues from different plant species subjected to abiotic stress.

Plants tolerate many variable environmental conditions during growth and development. Efforts have been made to understand molecular pathways involved in plant responses to adverse conditions. Desikan et al. (2001) subjected *Arabidopsis* to oxidative stress and found an increase in genes involved in cell rescue and defense as well as other metabolic functions from the H_2O_2 treatment. Approximately 34 % were of unknown gene function, 18 % were involved in transcription, 18 % were involved in cell response or defense, 12 % were involved with metabolism, 8 % were involved in cellular organization and biogenesis, 6 % were involved with signal transduction, 3 % were involved with protein destination and transport and 1 % were involved with energy production. Kreps et al. (2002) studied expression profiles in the leaves and roots of *Arabidopsis* subjected to hyperosmotic, salt, and cold conditions. Kreps et al. (2002) showed the majority of the transcriptome changes were stimulus specific and distinct sets of genes were expressed during hyperosmotic, salt, and cold stress. During the initial phase of the stress response, less than 5 % of transcript changes were shared by all three stress conditions and the number of genes common between all three stress conditions decreased the longer plants were exposed to the conditions. Seki et al. (2001) used full-length cDNAs from *Arabidopsis* grown in different conditions and from various developmental stages from germination to mature seed. Seki et al. (2001) isolated a group of genes involved with drought, some which were not previously identified as drought responsive. A more detailed analysis with *Arabidopsis* genomic sequences indicated that 12 of the genes isolated were regulated by the dehydration responsive element (DRE) which has been previously reported as essential for the expression of drought-responsive genes (Yamaguchi-Shinozaki and Shinozaki 1994). Such an approach may offer new information about *cis* acting elements and the transcription factors that bind to them.

Watkinson et al. (2003) measured changes in gene expression of loblolly pine exposed to both mild and severe drought stress. Water was withheld from rooted plantlets at -1MPa and -1.5 MPa for mild and severe stress, respectively. Net photosynthesis was measured for each level of stress and RNA isolated from the needles to estimate photosynthetic acclimation in response to drought stress. Under mild stress, loblolly pine showed a reduction in photosynthetic rate followed by recovery to near control levels in subsequent drought cycles. The microarray analysis consisted of cDNA clones from 5 pine EST libraries and gene expression profiles were correlated

with physiological data reflecting photosynthetic acclimation to mild stress and photosynthetic failure due to severe stress. Greater changes in gene transcript levels under mild stress was associated with physiological acclimation. The number of clones showing either positive or negative changes in transcript levels increased from 5.8 % to 8.6 % between mild cycles 1 and 2 suggesting the behavior of these genes was correlated with acclimation. The number of genes showing negative changes indicates the importance of down regulation as well as up regulation in acclimation to drought stress. No comparable changes occurred when plants were grown under severe water deficit. Group 2 LEAs, flavanoid enzymes and genes associated with mitochondrial electron transport were upregulated during photosynthetic acclimation under mild drought stress. Carbon metabolism enzymes, that provide carbon compounds to other metabolic processes, pyruvate kinase and pyruvate dehydrogenase, increased during mild stress. During severe stress, reduced transcript levels of genes associated with the reductive pentose phosphate pathway was observed, suggesting decreased photosynthesis which was correlated with decreased photosynthetic rate. Regulation of genes associated with polyamines involved in senescence and dormancy were observed.

A number of microarray analysis projects have been published that isolated and characterized genes involved in cold acclimation. Three studies were on *Arabidopsis,* which has limited hardiness (Seki et al., 2001; Fowler and Thomashow 2002; Kreps et al., 2002) and one on sugarcane (*Saccharum sp*), a chilling sensitive plant which has no potential to cold acclimate (Nogueira et al., 2003). Fowler and Thomashow (2002) treated *Arabidopsis* plants for 7 days at 4 °C and profiled the expression patterns of approximately 8000 cDNAs using micro-array analysis and found 306 genes were cold responsive with 218 genes up regulated and 88 genes down-regulated. Of the genes that were up-regulated, 64 genes were expressed during the cold treatment and 156 genes were transiently expressed. Over 50 genes expressed during the cold treatment had not been previously identified as cold responsive. Seki et al. (2001) studied the expression patterns of approximately 1300 full length cDNAs isolated from *Arabidopsis* under control, drought and cold temperature treatments against cDNA isolated from transgenic and wild type *Arabidopsis* and demonstrated 19 genes induced by cold, of which 10 genes were not previously identified as cold responsive. Nogueira et al. (2003) identified numerous cold inducible ESTs, from sugarcane showing a chilling sensitive plant has the ability to respond to acclimating temperatures.

The majority of work regarding cold acclimation has been conducted in cereals and *Arabidopsis*. *Arabidopsis* is ideal to study because it is a small plant with a short life cycle and a small genome that has been recently sequenced (The *Arabidopsis* Genome Initiative 2000). Much knowledge has been gained from *Arabidopsis* and as a result comparisons have been made with *B. napus*, to determine if differences in gene regulation exist (Weretilnyk et al., 1993; Girke et al., 2000). *B. napus* is an economically important crop grown for its edible oil. In Canada, 6,669,000 tonnes of canola was produced in 2003 (Statistics Canada, 2003) worth approximately 2.3 billion dollars to the

producer. An increase in freezing tolerance would have a dramatic effect on the sustainability of the crop. An increase in freezing tolerance of 2-3 ºC would protect seedlings from episodic spring and fall frosts that reduce seed yield and oil quality. We used a proprietary gene chip microarray developed from *B. napus* to determine transcript expression during cold acclimation of a spring and winter variety of *B. napus* in controlled conditions and in autumn field conditions. Winter canola, in contrast to spring canola, has the ability to survive extended periods of cellular dehydration due to sub-zero temperatures. A statistical data mining analysis was conducted to compare gene expression during cold acclimation with level of freezing tolerance. Genes were selected based on relative expression in 2 low and 2 high freezing tolerance samples using a selection difference of log10 = 0.5 and correlation analysis conducted of gene expression profiles positively correlated and negatively correlated with level of freezing tolerance with $r > 0.4$ or $r < -0.4$, respectively. Those genes that had a significant positive or negative correlation (at 5% probability) with level of freezing tolerance in all controlled environments were reported. The analysis found the expression of 99 genes positively correlated and 71 genes negatively correlated with increased freezing tolerance.

Contrasts based on the results of the microarray analysis indicate that initial exposure to acclimating temperature, a greater number of genes increased expression in the leaf material of the winter type compared to the spring type. At the end of cold acclimation, a greater number of genes decreased expression in the spring type compared to the winter type. Furthermore, at the end of the cold acclimation regime, more than 50 % of the genes observed to increase or decrease expression appeared to be different between both canola types, even though both types increased freezing tolerance at similar rates. A greater number of genes appear to be up-regulated in the winter compared to the spring type canola with the onset of cold acclimation.

Under controlled environment conditions (indoor), a greater number of genes increased expression in the acclimated spring type compared to the winter type, however, when field acclimation was compared to indoor acclimation, genes that increased expression were similar between the two types. In the microarray analyses, spring and winter canola acclimated under field conditions had the same level of freezing tolerance as spring and winter canola acclimated under controlled environment conditions. The levels of freezing tolerance achieved by the spring and winter types indicate that the two canola types responded similarly under field and controlled conditions. Gusta et al. (1997) reported that winter rye has the potential to acclimate to -33 °C under field conditions and under controlled environment conditions, acclimate to -28 °C. Gusta et al. (1997) reported winter wheat acclimated to -23 °C under field conditions and controlled environmental conditions. To study this difference further, Gusta et al. (2001) compared the freezing tolerance of thirty-three winter wheat cultivars acclimated in soil under natural field conditions and controlled environment conditions. The winter wheat variety Norstar increased its level of freezing tolerance to approximately -24 °C in both natural and controlled environment conditions as observed previously in Gusta et al.

(1997). However, cultivars that did not show increases in freezing tolerance beyond -17 °C under field conditions were generally capable of cold acclimating further under controlled environment conditions (Gusta et al., 2001).

The results from the microarray analysis revealed the expression patterns of genes during cold acclimation and identified potential transcription factors. The use of the winter and spring canola types allowed physiological comparisons at key changes in temperature and isolation of genes not observed in each type. These results can be applied to comparative and functional genomics. Comparative genomics allow for phylogenetic analysis of gene families to identify putative orthologs to genes characterized in *Arabidopsis* or other plant species, providing information regarding gene function. Genomic sequences allow for identification of *cis* regulatory elements based on phylogenetic conservation and similarity in gene expression. Functional genomics can be used to study and understand how *B. napus* responds and adapts to abiotic stress. Cellular and physiological aspects of gene function in canola can be made when canola is grown and acclimated in both controlled environment conditions and field conditions. The function of gene sequences highly correlated to with cold acclimation can be studied using transgenic approaches to achieve overexpression or silencing of specific genes.

4. PROTEOMICS

The induction of cold-induced proteins have long been associated with the development of freezing tolerance (Levitt, 1980). The term “proteomics” is used to describe the study of alterations and interactions that occur in all the proteins of a plant (i.e., its ‘proteome’). Current research employs highly sophisticated equipment and procedures; such as two dimensional polyacrylamide gel electrophoresis (2D PAGE) (Blackstock and Mann, 2000), differential gel expression (DIGE, Amersham Biosciences) (Swatton et al., 2004) and multidimensional protein identification technology (MudPIT) (Yates, 1997) for the separation of proteins based on different chemical characteristics. Highly sensitive mass spectral technology is used to identify each protein and high capacity computers are used to search the ever expanding databases to determine qualitative and quantitative changes in protein accumulation patterns.

The term ‘proteomics’ is a relatively new term to describe the study of protein-expression profiles within an organism, but in actuality protein research has been conducted for the past 100 years. Many studies have been conducted to characterize enzyme variation, protein content and protein synthesis in plants exposed to low temperature (LT) (Guy, 1990). As early as 1969, cold research scientists realized that many biochemical and genetic changes were occurring within plants that were exposed to low temperature (McGown et al., 1969). To characterize these biochemical changes, freeze stability and isozymic variation of enzymes were measured in non-acclimated (NA) and acclimated plants (AC) (McGown et al., 1969). Levels of peroxidase activity was measured in NA and AC plants of four unrelated woody species, revealing that three of four

species had measurable peroxidase activity in only AC plants. Similar measurements were conducted for invertase in NA and AC wheat, identifying that alterations in protein structure occurred as a result of cold acclimation (Roberts, 1974). The altered form of the enzyme present in CA wheat exhibited different kinetic properties (i.e. was more efficient at LT) and therefore functionally replaced the lower molecular weight form present in NA seedlings (Roberts, 1978). Similar alterations in enzyme conformation were also observed in fully acclimated alfalfa plants, where shifts in the structure of several dehydrogenases involved in the respiratory pathway enabled them to function at LT (Krasnuk et al., 1975, 1976, 1976a). Based on this discovery the activity, structure and stability of many enzymes were measured in NA and AC tissues of a multitude of species with similar results (Huner et al., 1976, 1978, 1979, 1981; Griffith et al., 1985).

In the late 1940's it was realized that total protein content changes in response to LT when levels of soluble proteins were measured in bark cells of black locust (*Robinia pseudoaccia L*) (Siminovitch and Briggs, 1949). In this study, total soluble protein content was higher in fully acclimated bark. To expand this study, measurements were taken at each season in a year, demonstrating that total protein content increases in autumn closely paralleling increases in freezing tolerance (Siminovich and Briggs, 1953). Protein levels were maintained over the winter months dropped as the temperature increased in spring and dormancy was broken. Seasonal changes in total protein content have since been reported in many other species (Parker, 1962; Coleman et al., 1966; Gerloff et al., 1967; Pomeroy et al., 1970; Chen and Li, 1980; and Guy and Haskell, 1987) similar but not universal responses have been observed. These studies also led to the realization that protein changes in response to low temperature exposure were not only quantitative but also qualitative (Siminovich and Briggs, 1953), as it was realized that the proteins synthesized in plants exposed to LT were not identical to those isolated from NA tissues (McGown et al., 1968; Cracker at al., 1969; Davis and Gilbert, 1970; Faw and Jung, 1972; Brown and Bixby, 1975; Faw et al., 1976; Huner at al., 1976; Kacperska-Palacz et al., 1977; Rosas et al., 1986). The general consensus of these studies indicated that a subset of LT proteins were synthesized in addition to those present in NA tissues, but these were difficult to detect with present day electrophoretic methods.

As early as 1970, Weiser hypothesized that cold acclimation resulted in the expression of a novel set of genes not expressed in NA plants (Weiser, 1970). This hypothesis has since been proven in a variety of independent studies (Marmiroli et al., 1986; Yacoob and Filion, 1986, 1986a; Meza-Basso et al., 1986, Johnson-Flanagan and Singh, 1987; Guy and Haskell, 1987, 1988; Johnson-Flanagan and Singh, 1987; Laroch and Hopkins, 1987; Mohapatra et al, 1987; Ougham, 1987; Robertson et al., 1987; Sarhan and Perras, 1987; Tseng and Li, 1987; Cooper and Ort, 1988; Kurkela et al., 1988; Hahn and Walbot, 1989; Perras and Sarhan, 1989). Careful examination of the proteins that accumulate in response to LT exposure in many different species has revealed the appearance and decline of similar classes and types of proteins. These proteins are, however, significantly less conserved than those that arise as a result of heat shock

(Barnett et al., 1980; Key et al., 1981). Unlike the heat shock response, CA material continues to accumulate 'housekeeping' proteins (Marmiroli et al., 1986; Yacoob and Filion, 1986, 1996a; Guy and Haskell, 1987; Gilmour et al., 1988; Guy et al., 1988; Perras and Sarhan, 1989), as CA is a process that requires energy and continued metabolism (Guy and Haskell, 1987).

With the discovery of new molecular techniques and the improvement of existing techniques, changes in gene expression were commonly quantified via the detection of mRNA levels. Recent evidence has shown that transcript expression data does not always correlate with protein accumulation patterns (Whitelegge, 2002). In order to accurately identify and quantify changes with in a cell or organism it is now believed that one must surpass the transcript and look directly at the protein level. The systematic analysis of proteins present within the plant at any given period is referred to as proteomics (van Wijk, 2001). Proteomics is currently the leading technology for the high throughput analysis of protein accumulation patterns. Applications of proteomic analysis include the identification of proteins that accumulate within a cell or organism, the analysis of post translational modification of proteins and the interaction of proteins within a living system (protein-protein interactions)(Whitelegge, 2002). The mass identification of proteins, commonly referred to as the 'Brute Force' method most commonly occurs via two dimensional electrophoresis (2DE), which uses immobilized pH gradient strips to separate proteins in the first dimension (based on pI) and SDS-PAGE in the second dimension to separate proteins based on molecular weight (MW) (Blackstock and Mann, 2000). Following separation, gels are typically analyzed with the assistance of computer programs for changes in accumulation patterns, and differentially accumulated proteins are robotically isolated from the gel. Isolated proteins are then digested (typically via trypsin) into peptide fragments and the masses are determined by LC MS/MS (Blackstock and Mann, 2000). Once masses data are obtained, they are compared to databases and subsequently identified. This method of protein identification has only recently become efficient, as a result of the large quantities of sequence data generated by early generation genomics programs (Rowley et al., 2000). As with all highly technical analysis, 2D-PAGE does possess limitations that involved problems with dynamic resolution (Blackstock and Mann, 2000), quantification and identification of low abundance and hydrophobic proteins (van Wijk, 2001). To reduce the problems associated with 2DE, a new technique that involves differentially labeling protein samples with fluorescent dyes has been developed (Amersham Biosciences). This technique, referred to as DIGE, uses the same principles as 2D-PAGE except multiple protein samples (labeled with different florescent dyes) are analyzed within one gel. By analyzing all samples within the same gel, gel to gel variation is eliminated resulting in increased quantification and dynamic range accuracy (Swatton et al., 2004).

A different high throughput method for protein isolation and quantification requires the utilization of multidimensional separation methods, that are different but complimentary to those used in 2D-PAGE (pI and MW). This method is referred to as multi-dimensional protein identification technology (MudPIT) and it generates pep-

tides from complex protein mixtures (whole cell) that have been first separated in the first dimension by a strong cation exchange column followed by reverse phase chromatography (RPC) in the second dimension. Throughout the process RPC fractions containing peptides with different chemical characteristics are collected and fed directly into a MS. Due to the direct link of the PRC to the MS, this system provides a very efficient high throughput method of protein isolation. As a result it is possible to detect and quantify low abundance proteins that would be difficult via 2D-PAGE. Although MudPIT provides a more efficient method for the mass identification of proteins compared to 2D-PAGE, it also possesses limitations. The first limitation is that this process of protein identification and quantification is sequence dependent (van Wijk, 2001). Labeling of proteins for ICAT ion exchange requires a cysteine residue, which is not universally present in all proteins (e.g. myoglobin). As a result a certain quantity of peptides are not labeled and are therefore missed. The opposite effect can occur in peptides that have large numbers of cysteine residues present in their sequence, as proteins possessing too many labels are difficult to quantify (Blackstock and Mann, 2000). Another major limiting factor of MudPIT is that it is not capable of differentiating between modified forms of the same protein (e.g. post translational modifications)(Gygi et al., 1999). In instances where post translational modifications have been made to a protein, there is now way to visualize this with MudPIT as is possible in gel electrophoresis

To date there are few published results on high throughput proteomic analysis on plant material that has been exposed to low temperature. In one study 2D-PAGE was utilized to isolated nuclear proteins from the nuclei of *Arabidopsis* that were exposed to low temperature (Bae et al., 2003). Out of a total of 184 proteins identified in the nuclear proteome, 54 were found to increase a minimum of two-fold in response to the cold. Of these 54 proteins a few notables included a 60s ribosomal protein, a transcriptional regulator, a DEAD box RNA helicase and an HSP-70. In another study, utilizing 2D-PAGE, a wide spread proteomic analysis of *Brassica napus* has revealed that approximately 100 proteins accumulate or disappear at some point in the cold acclimation process in a winter and a spring variety of canola (Trischuk and Gusta, unpublished results). Some of the more interesting proteins identified included carbohydrate metabolism enzymes, free radical scavenging enzymes as well as many proteins that possessed homology to late embryogenesis abundant (LEA) proteins which have been associated with the development of freezing tolerance. Another interesting discovery from this research is that the set of proteins that accumulate upon initial exposure to LT (LT_{50} -7°C) are very different than those that are present once the plants have become fully acclimated (LT_{50} -15°C).

Despite the lack of large scale proteomics data on cold treated plant material, a great deal of work on proteins has been accomplished. To date thousands of publications have been written, listing a tremendous variety of proteins that are induced by LT or involved in the cold acclimation process. The majority of the proteins identified fall into a few distinct classes based on function. The first major class are the LEA-D11

(dehydrin) proteins (Close, 1997), which are induced in response to cold as well as several other abiotic stresses. Many of these proteins accumulate during CA, and are hypothesized to interact with membranes and proteins, stabilizing them under desiccation conditions (Wolkers et al., 2001). Well noted examples include the dehydrins (DHNs) from barley (Close, 1997), cold responsive (COR) proteins from *Arabidopsis* (Gilmour et al., 1992), wheat cold specific (WCS) (Houde et al., 1992) and wheat cold responsive (WCOR) (Danyluk et al., 1998) from wheat. Another group of proteins that are induced in response to LT exposure are antioxidant enzymes that are responsible for scavenging reactive oxygen species produced in response to environmental stress (Wu et al., 1999). Examples of these types of enzymes include superoxide dismutase (SOD) (McKersie et al., 1993), ascorbate peroxidase (APX), and glutathione reductase (Sen Gupta et al., 1993). A method to minimize the number of proteins in a sample, is to fractionate into sub-samples based typically on a specific chemical characteristic (e.g. soluble vs. insoluble) or in most cases based on an organelle within the cell (e.g. ribosome, chloroplast, membrane, etc.). Although the extracellular spaces are not considered an organelle, a complete proteomic study of the proteins present in this space has been accomplished (Griffith et al., 1992). Within the extracellular spaces of a plant, a group of antifreeze proteins (originally isolated from flounder (DeVries, 1971) are present. These proteins are classified based on their ability to alter the crystal structure of ice, and inhibiting the formation of secondary ice crystals (Griffith et al., 1992). In total seven proteins were identified in the apoplastic spaces, with five of them showing antifreeze properties. Proteins identified in LT exposed plants include some examples associated with photosynthesis (Gray et al., 1997) and carbohydrate production (Olien and Clark, 1993), membrane associated proteins (e.g. calcium transport proteins) (Monroy et al., 1993), and signal transduction proteins (e.g. protein kinases, MAPKs, etc) (Monroy et al., 1993a). Although the term proteomics is quite novel, the amount of research conducted on protein accumulation in low temperature tolerant plants is quite significant. It has been well established that proteins accumulate in response to cold, and that proteins present in CA plants are well adapted for efficient activity at LT. Due to the inefficiency of molecular techniques, this is about all we know; with the advent of new technical techniques like 2D-PAGE, DIGE and MuDPIT we will soon know much more.

5. METABOLIC PROFILING

Major metabolic shifts occur with the development of cold acclimation (Levitt, 1980). The metabolism of glucose-6-phospate shifts from glycolysis to the pentose pathway to generate NADPH and nucleic acid precursors (Sagisaka, 1974). Reducing power utilizing reactions are favoured with the generation of ascorbic acid (Andrews and Pomeroy, 1978), glutathione (Guy and Carter, 1982) and reduced pyridine nucleotides (Kacperska, 1985). Adenylate energy charge, ATP, $NADPH_2$ which are required for metabolism and repair are elevated during cold acclimation of *Brassica* leaves (Kacperska, 1999). Major changes in osmotic potential are related to sugars and sugar

alcohols which increase in the autumn during acclimation and decrease in the spring during deacclimation (Levitt, 1980). Many wheat and canola cultivars show a parallel relationship between sugars and freezing tolerance. The main sugars that increase are sucrose, glucose, fructose, sorbitol, manitol, raffinose, and stachyose. It is postulated sugars replace water and decreases the degree of freeze-induced dehydration. Trehalose and umbelliferose have received considerable attention in the stress response as they promote glass transitions that protect cells from desiccation injury (Crowe et al., 1984, Wolkers et al., 1999). Stress induced proteins have a stabilizing effect on sugar glasses by increasing the average strength of hydrogen bonding in the dry state (Wolkers et al., 2001). Long chain fructans increase during cold acclimation of cereals and inhibit ice growth in xylem vessels (Olien, 1967). Thylakoid membranes are protected from freezing inactivation by exogenous proline, argenine, threonine and lysine. Proline and glycine-betaine are both postulated to act as cryoprotectants. Yoshida and Uemura (1984) observed the development of freezing tolerance was accompanied by an increase in phospholipids, especially phosphatidyl choline and phosphatidyl ethanolamine. Free fatty acids were shown to accumulate as degradation products from ROS following a lethal-thaw event (McKersie and Bowley, 1996).

Previously, it was impossible to measure all these changes simultaneously; however, ultra-high resolution mass spectrometry can identify and quantify over 100,000s of metabolites, simultaneously. The number of metabolites that can be identified and quantified depends on the sample preparation x instrument sensitivity x physical concentration of the metabolite. Subtle differences in expressed genes and proteins can be verified through metabolomics.

6. HORMONAL PROFILING

In autumn, plants in the field are exposed to multiple stresses that play a role in determining their capacity to survive the winter. These stresses include low temperatures (both above and sub-zero), wind, drought, UV, photoinhibition, nutrients, salinity, high temperatures and mechanical injury. Plants use multiple signaling pathways and signals to mediate their acclimation responses. Two signaling pathways have been speculated to regulate cold acclimation (Thomashow, 1999); however, it is the specific combination of various components of the signaling network coupled with spatial and temporal factors that ultimately result in an increase in winter hardiness. Low temperature sensors may be due to changes in membrane fluidity (Murata and Los, 1997), conformational changes in proteins, altered ABA binding sites, release of sequestered ABA from plastids, decrease in cell water potential, etc. Secondary signals such as ABA and second messengers can initiate a cascade of signaling events that may differ from the critical primary signal. For a comprehensive review on cell signaling the reader is referred to Xiong et al., (2002). Calcium has been demonstrated to act as a secondary messenger in low temperature signal transduction during cold acclimation (Monroy and Dhindsa, 1995). These studies are based on the observation of a transient increase

in cytosolic Ca^{2+} when the temperature is lowered to induce cold acclimation (Knight, 2000). However, this phenomenon is not conclusive as it is only demonstrated under a fast cooling rate and not if the temperature decrease is less than 10ºC in one hour (Plieth et al., 1999).

Many aspects of plant development, including stress tolerance, are regulated by antagonistic interactions between plant hormones e.g. abscisic acid (ABA) and gibberellins (Gomez-Cadenas et al., 2001); auxins and cytokinins (Coenen et al., 2003); and by synergistic interactions e.g. ABA and jasmonic acid (JA) (Wilen et al., 1994). Abscisic acid a growth inhibitor, induces cold acclimation in a wide range of plants (Chen and Gusta 1983, Reaney et al., 1989), whereas gibberellins are growth promoters and counteract the effect of ABA (Reaney et al., 1989). Recent analysis of Arabidopsis mutants demonstrated strong interactions between ABA and other signalling pathways, including auxin, sugar and ethylene (Lu and Fedoroff, 2000, Gazzarrini and McCourt, 2003). Arabidopsis mutants have been identified that show reduced sensitivity to both ABA and auxins (Suzuki et al., 2001, Monroe-Augustus et al., 2003). There is substantial evidence implicating MAPK pathways in both ABA and auxin signalling.

There is a great deal of confusion regarding the role of ABA in the development and maintenance of freezing tolerance and this has led to the suggestion that there are two pathways; an ABA independent pathway and an ABA dependent pathway. This evidence is based on the finding that ABA at non acclimating temperatures fails to up regulate several cold associated genes. It has been assumed that all cold upregulated genes respond similarly, and therefore, genes that do not respond to exogenous ABA, but only to low temperatures are independent of ABA. Few attempts in these studies have made the effort to establish the uptake of ABA and its translocation to the site of interest or if ABA is absorbed by the cells in question. Foliarly applied ABA is not readily absorbed in contrast to a root drench application. Often ABA mutants that are either insensitive to ABA or are deficient in ABA are used to determine the role of ABA in the stress response. In many of these studies using ABA insensitive mutants the quality of exogenous ABA taken up by the cell in question has not been determined, or if exogenous ABA has been degraded or sequestered or if non-stress ABA responsive genes have been upregulated or if the temperature is conducive to the binding of ABA at its receptor site. ABA deficient mutants have reduced levels of ABA in comparison to the wild types. The limitation of these mutants of the optimum concentration for gene regulation is not known and therefore these mutants are always "leaky". There are very specific, highly quantitative tests available to measure ABA (Chiwocha et al., 2001) in contrast to the less specific ELISA tests. In addition there are at least three unique pathways for the synthesis of ABA (Zhou et al., 2004). Thus a mutation in one pathway may not necessarily affect another pathway. Therefore there is an inherent ambiguity in using these mutants to establish the role of ABA in stress. Other possibilities for lack of response to ABA is degradation, sequestration and unavailability of ABA binding sites. Often ABA acts in concert with other

phytohormones such as auxins as described above (Lu and Fedoroff, 2000) or in combination with sucrose (Rolland et al., 2002). Wilen et al. (1994) observed brome-grass suspension cells grown at 20°C possessed little freezing tolerance in the presence of R-ABA (the unnatural form) but were extremely freezing tolerant in the presence of S-ABA (the natural form). Both enantiomers up regulated similar cold associated genes; however, the major difference was S-ABA enhanced the uptake of sucrose from the medium, whereas R-ABA did not. It is well documented an increase in sucrose concentration during cold acclimation is intimately associated with the development of freezing tolerance (Levitt 1980) and a combination of both stress associated proteins and sucrose confer stress protection (Robertson et al. 1994). In addition, sucrose and other sugars play a central role as signalling molecules that modulate the physiology, metabolism and development of plants. (Koch, 1996, Coruzzi and Zhou, 2001, Arroyo et al., 2003). There is ample evidence that several stress related genes contain sugar boxes, that regulate expression (Atanassova et al., 2003) and that this sugar induction is enhanced strongly by ABA (Cabir et al., 2003). Rook et al., (2001), working with two sugar-induced genes involved in starch biosynthesis, demonstrated ABA strongly enhances their sucrose-induced expression but has no effect in the absence of sucrose. Wanner and Junttila, (1999) reported in the dark cold associated genes were upregulated at low temperatures, but the plants did not increase in freezing tolerance. However, in the light plants increased freezing tolerance with the accumulation of both sucrose and upregulation of stress associated genes. The old school of thought assumed that elevated levels of phytohormones were required to elicit gene changes; however, recent evidence does not support this contention (Chiwocha et al., 2003). Since freezing tolerance is a multigene event it is rather presumptuous to assume the single addition of a phytohormone at non acclimating temperatures to actively growing plants would have a major effect on cold acclimation.

The GA/ABA antagonism in regulating development is well established (Chrispeels and Varner 1966, Gomez-Cadenas et al., 2001). The effect cytokinins have on the development of freezing tolerance is poorly understood (Reany et al., 1989). Cytokinins under non-stressful conditions promote growth and development. Under stressful conditions, cytokinins are degraded by cytokinin oxidase, which is induced by ABA (Brugiere et al., 2003). The balance between auxins and cytokinins controls the formation of roots, shoots (Skoog and Miller, 1957), the outgrowth of shoot auxiliary buds (Sachs and Thimann, 1967) and the formation of lateral roots (Wightman et al. 1980). Roots, the most sensitive tissue of plants, are killed between -6°C to -10°C and do not survive overwintering and must be regenerated in the spring, when the seedlings are recovering from overwintering injury (Chen et al. 1983).

Both auxin and cytokinins rapidly induce ethylene synthesis in many tissues (Abeles 1966, Vogel et al., 1998); however, cytokinins in certain species inhibit ethylene production (Coenen et al., 2003). Abiotic stress activates a MAPK cascade, (Sangwan et al., 2002) which increases intracellular calcium (Moyen et al., 1998), calmodulin (Bergey and Ryan 1999), and the activation of phospholipase A_2 (Lee et al., 1997).

Phospholiphase releases membrane linolenic acid, the precursor to oxylipins and JA (Bergey et al., 1996). The combination of oxylipins, JA and ethylene act synergistically to induce the expression of stress associated genes (O'Donnell et al., 2003). Abiotic stress results in a JA mediated induction of wound related genes and the synthesis of proteinase inhibitors (Conconi et al., 1996), which are also activated by water deficit, salinity and ABA (Chao et al., 1999). Salicylic acid (SA), which plays a key role in plant disease resistance and hypersensitive cell death, is involved in acclimation to abiotic stresses. Exogenous SA increased abiotic stress tolerance by reducing reactive oxygen species (ROS) in bean and tomato (Ding et al., 2002), as well as increasing cold tolerance in wheat (Gusta unpublished results). Glutathione S-transferase (GST) genes code for glutathione, which is involved in the binding and transport of hormones and in the reduction of ROS (Edwards et al., 2000). It is interesting to note that the GST genes are also activated by auxins and JA (Chen and Singh, 1999). Glutathione, acting as a ROS scavenger, has long been implicated in freezing injury (McKersie and Bowley, 1996). McKersie et al., (1996) were among the first to propose death of winter annuals was caused by the formation of ROS during prolonged periods of freeze-induced dehydration. Thus, hormones comprise a very complex network of signalling molecules at the cellular level. This has led to the suggestion that phytohormone responses cannot be reduced to simple linear pathways that connect inputs and outputs but are more probably by interactive networks (Moller and Chuaa, 1999, Gazzarrini and McCourt, 2003).

To date, a complete analysis of phytohormonal involvement in cold acclimation has not been performed. Recently, a highly sensitive and selective method for the simultaneous profiling and quantification of a wide variety of plant hormone groups and their metabolites using high-performance liquid chromatography (HPLC) coupled with electrospray ionization-tandem mass spectrometry (ESI-MS/MS) has been developed (Chiwocha et al., 2003). Each compound is analyzed in its native state without the need for derivatization. High temperatures are not required since the compounds are separated by HPLC and the plant hormones and metabolites are analyzed using either positive or negative ion electrospray in a single LC-MS/MS run. To date, over 20 compounds with hormonal activity can be analyzed and quantified simultaneously.

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CHAPTER 6

PHOTOOXIDATIVE STRESS

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1. INTRODUCTION

Plants are exposed to several environmental stresses, that adversely affect metabolism, growth and yield. Yet, plants are also known to adapt to these stress conditions by modulating their metabolism and physiology. These stress factors include abiotic (drought, salinity, light, CO_2, soil nutrients and temperature) and biotic (bacteria, fungi, viruses and insects) components. Among abiotic factors, non-optimal light intensity and temperature can be considered as the most serious limiting factors which limit the growth and yield of plants (Foyer, 2002; Reddy et al., 2004). Also, environmental fluctuations often result in 'stress' which ultimately limit the overall plant performance. The consequences of environmental stresses on the whole plant are quite complex, dealing with structural and metabolic functions. Understanding plant responses to the external environments is of greater significance for making crops stress tolerant. One of the most deleterious effect of environmental stress on plants is "oxidative stress" in cells, which is characterized by the accumulation of potential harmful reactive oxygen species (ROS) in tissues. Photooxidative stress in plants is mostly induced by the absorption of excess excitation energy leading to over-reduction of the electron transport chains generating ROS.

Although excess light absorption is known to cause photooxidative stress, paradoxically photo-chilling, salinity and drought are also responsible in inducing photooxidative stress in plants (Asada, 1999; Foyer and Noctor, 2000; Reddy et al., 2004). This review concentrates on recent developments on the effects of light stress- induced oxidative responses in plants. We focus on various physiological, biochemical,

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biophysical and molecular responses in plant cells under photooxidative stress. Special emphasis is given on chloroplast processes under excess light regimes.

Plants are under stress when the available light is either in excess or limiting. The present article deals only with the response of plants to excess light. Readers interested in the topic of low light stress or phenomena such as sun-flecks may refer to relevant reviews (Pearcy, 1998; Noctor et al., 2002). The phenomenon of photooxidative stress develops not only under supra-optimal light but also at normal light when the biochemical reactions are limited by sub-optimal levels of temperature, water or nutrition. There are also several excellent reviews on the topic of photoinhibition and oxidative stress (Foyer et al., 1994; Ort, 2001;Oquist and Huner, 2003) besides few books (Pearcy, 1999; Das, 2004; Demmig-Adams et al., 2005)

2. LIGHT USE BY PLANTS

Light is the ultimate energy source for photosynthesis and it is also one of the most deleterious environmental factors causing photooxidative stress in plants (Asada, 1999). Less than 1% of the 1.3kWm^{-2} solar energy reaching the earth is absorbed by plant tissues and is used in the synthesis of energy-rich biomolecules (Salisbury and Ross, 1992). It is estimated that 3 x 10^8 kJ of chemical energy derived from sunlight per year are fixed globally in the form of 2x10^{11} tons of fixed carbon. Photosynthesis is the only basic energy-supplying process on the earth. Leaf photosynthetic capacity (rate of photosynthesis per unit leaf area) differs greatly for species living in diverse habitats in both tropical and temperate climates. Many crops have photosynthetic efficiencies ranging from 0.1% to 3%, since only 0.83 kWm^{-2} (64%) of the total 1.3 kWm^{-2} radiant energy reaching the earth is in the PAR region (McDonald, 2003). Plants are known to adapt to a wide range of light environments ranging from deep shade of rain forests to high radiation environments of deserts and mountain tops. The leaves of shade plants exhibit morphological and anatomical features which differ from plants growing in sunlight. Shade plants have more chloroplasts than sun leaves while the latter become thicker than the shade leaves due to longer and for additional palisade cells (Bjorkman and Powels, 1981). Mohr and Schopfer (1995) reported that tomato plants have hundred stomata per mm^2 on the lower epidermis in low light. However, when the plants were transferred to high light the leaves developed more number of stomata within three days of a change in the light condition.

Plants have evolved mechanisms protecting against photodamage which include chloroplast movements that reduce light exposure for the organelle and photosynthetic complexes (Haupt, 1990) and leaf movement or paraheliotropism to avoid light and heat (Ludlow and Bjorkman, 1984; Pastenes et al., 2004). Paraheliotropism is known to result from an osmotic change at the pulvinus and this phenomenon confers protection against photoinhibition and maintains leaf temperature well below air temperatures (Assman, 1993). Light absorption can also be regulated at the tissue and organelle level and accordingly, isobilateral and dorsiventral leaves are known based

on the distribution of the photosynthetic cells, as well as density and location of chloroplasts within the leaves for optimized capture of light energy (McDonald, 2003). High light stress can induce photoinhibiton, photoactivation, photodamage and degradation of photosynthetic proteins in plant cells (Deming-Adams and Adams, 1992; Long and Humphries, 1994; Jiao et al., 2004). Such excess light conditions might arise from high irradiance and in concert with other stressful conditions such as drought and high or low temperatures.

3. PHOTOOXIDATIVE STRESS

The light dependent generation of active oxygen species is termed as photooxidative stress (Foyer et al., 1994). During the life cycle of plants, they are exposed to varying light environments and plants develop several acclimation responses. Evolution has refined the photosynthetic apparatus for high photosynthetic efficiency in limiting light with regulatory features to ensure that light intensities can be endured without photodamage (Ort and Baker, 2002). Miyake and Vokata (2000) indicated that high growth irradiance enhances the electron partitioning to O_2 at PSI. Golden variety of tropical fig, *Ficus microcarpa* showed hypersensitivity to strong light as it lacks heat stable dehydroascorbate reductase (DHAR), suggesting the crucial role of ascorbic acid (AsA) regeneration system for the tolerance against high irradiance (Yamasaki et al., 1999). Photorespiration also supplies electron acceptors to PSI and has a photoprotective role against the damage due to strong illumination (Kozaki and Takeba, 1996). Thus, the regulation of photosynthesis has been viewed as a dynamic balancing act in which photoprotection is reversibly traded for photosynthetic efficiency (Ort, 2001). The ability of plants to changing light environment allows them to achieve greater evolutionary success by growing under high irradiance intensity. Such variations in light environment may range from few seconds or minutes up to few or many days.

4. REACTIVE OXYGEN SPECIES

4.1. Singlet Oxygen ($^1O_2$) Generation

Under optimal growth conditions, light energy absorbed by the leaves is primarily used for carbon assimilation. However, when plants absorb more energy than is used in photosynthesis, they are subjected to photooxidative stress (Foyer, 2002; Krieger-Liszkay, 2005). Under such conditions, the light absorbed by the leaf can not be efficiently used for photosynthesis and becomes potentially damaged because the excess electrons react with the abundantly present oxygen. The relatively stable ground state of oxygen in a triplet state with the unpaired electrons is not directly a problem. However, under high light conditions, highly reactive singlet oxygen ($^1O_2$) can be produced by a triplet chlorophyll formation in the photosystem II (PSII) reaction center and in the antennae systems. Thus, the chlorophylls, in addition to use light energy in photosyn-

thesis, are also the potential sources of singlet oxygen ($^1O_2$) production. These reactive singlet oxygen molecules are generated by an input of energy by removing the spin restriction and therefore increasing the oxidizing ability of oxygen (Knox and Dodge, 1985; Niyogi, 1999). The half life time of $^1O_2$ is about 200ns in plant cells (Gorman and Rodgers, 1992). $^1O_2$ is known to react with DI protein, thus damaging PSII (Trebst et al., 2003). Keren et al. (2000) measured the degree of photoinactivation and loss of DI protein by using series of single turnover flashes. The highly reactive $^1O_2$ is also reported to have a strongly deleterious effect on chloroplast pigment-protein complexes, as it is generated in the pigment bed (Slooten et al., 1998; Niyogi, 1999). However, the DI damage is also regarded as physiological defense mechanism as the damaged DI protein is efficiently replaced by newly synthesized DI (Prasil et al., 1992; Aro et al., 1993). Suh et al. (2000) showed the production of $^1O_2$ in illuminated cytochrome b_6f complex by using spin trapping techniques. However, the role of cytochrome b_6f complex-generated $^1O_2$ is still not completely understood. However, it is now known that chlorophyll sensitizers act as main source of reactive oxygen species and in case the chlorophyll is activated by energy transfer under high light conditions, $^1O_2$ production is increased (Hippeli et al., 1999).

4.2. Photooxidation-Induced Free Radical Production in Plant Cells

High irradiance produces fluxes of dioxygen and excess electrons leading to over-reduction of electron transport chain (ETC), which might result increased formation of several free radicals, commonly referred as reactive oxygen species. Thus, high light-driven photosynthetic processes are main contributors to chloroplastic-ROS production in plants. Highly active ETC in chloroplasts under excess growth light operate in an O_2-rich environment and leakage of the excess electrons leads to the formation of ROS (Edreva, 2005a). Unlike the formation of $^1O_2$, chemical activation is the other mechanism to circumvent spin restriction through univalent reduction of dioxygen which results at least three intermediates namely superoxide ($O_2^{?-}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH·) (Figure 1). It is also known that these ROS colliding with an organic molecule may get an electron, rendering it a radical capable of propagating a chain reaction by forming peroxyl (ROO·) and alkoxyl (RO·) radicals (Perl-Treves and Peri, 2002). Excess electrons from ETC will be derived from ferridoxin to O_2. In addition, leakage of electrons to O_2 may also occur from 2Fe-2s and 4Fe-4s clusters of PSI. It is now well established that Q_A and Q_B sites of PSII are also potential sources of $O_2^{?-}$ generation (Dat et al., 2000; Zhang et al., 2003). The addition of an electron to molecular oxygen by photosynthetic ETC produces $O_2^{?-}$ and this reaction is termed as Mehler reaction (Mehler, 1951). The electron transfer to oxygen will be more at the chloroplast under high light stress because of high O_2 levels occurring at that site, favouring markedly high levels of $O_2^{?-}$ and $^1O_2$. We will now concentrate on the fate of this

disproportionate production of oxygen free radicals in plant cells under excess growth light regimes. Superoxide is capable of both oxidation and reduction. It can also react to produce several other reactive species. An enzyme, superoxide dismutase (SOD), present in the chloroplast matrix and in the thylakoid membrane dismutates superoxide to H_2O_2, particularly at low pH.

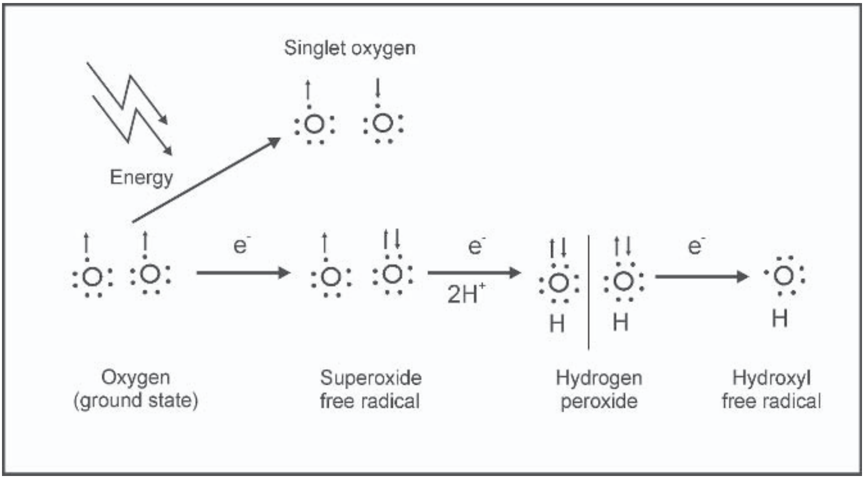

Figure 1. *Formation of reactive oxygen species from dioxygen*

$$O_2^{\cdot -} \xrightarrow[e^-,2H]{SOD} H_2O_2$$

H_2O_2 is not a free radical, but participates as an oxidant or reductant in several cellular metabolic processes. H_2O_2 is also produced in peroxisomes during photorespiration. When both superoxide and H_2O_2 are present at the same time a reaction catalyzed by transition metal ions, like iron and copper, favours the formation of toxic hydroxyl radical as shown in the following reaction known as Haber-Weiss reaction.

$$H_2O_2 + O_2^{\cdot -} \xrightarrow{Fe^{3+} \rightarrow Fe^{2+}} HO^{\cdot} + OH^- + O_2$$

The ROS are also produced in different cellular components including chloroplasts, mitochondria, peroxisomes, glyoxysomes, cell wall, plasma membrane and apoplasts (Figure 2). However, as depicted in Figure 2, the chloroplasts, mitochondria and the microbodies are the main sources of ROS in the plant cell.

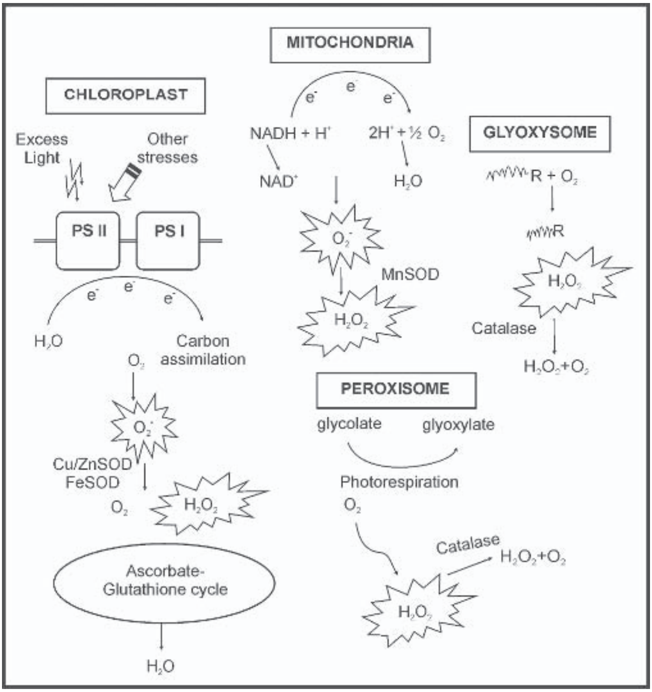

Figure 2. *Generation of reactive oxygen species in different cellular compartments*

Although chloroplast was considered to be the main source of ROS production, recent studies suggest some intriguing possibilities about other cellular organelles as additional sources of ROS generation. Plant hypersensitive response and programmed cell death were partly attributed to the enhanced levels of ROS in mitochondria (Lam et al., 2001). In plant cells, mitochondrial ETC is a major site of ROS production (Moller, 2001; Tiwari et al., 2002). In addition to the complexes I-IV, the plant mitochondrial ETC contains proton pumping alternative oxidases as well as two non-proton pumping NAD(P)H dehydrogenases on each side of the inner membrane. Complex I is the main enzyme oxidizing NADH under normal conditions and is also a major site of ROS generation (Figure 2). Several antioxidant enzymes are also reported in the matrix along with some antioxidants like glutathione to remove ROS produced under conditions of oxidative stress (Purvis, 1997; Braidot et al., 1999;). The entire ascorbate-glutathione cycle has been reported to occur in pea leaf mitochondria (Jimehez et al., 1998).

Cytosolic and apoplatic-ROS production have also been reported (Hammond-Kosack and Jones, 1996; Karpinski et al., 1997).

Photorespiratory production of H_2O_2 in peroxisomes is well known and the significance of peroxisomes in ROS metabolism is gaining recognition. Peroxisomes are not only the sites of ROS production by glycolate oxidase but also the site of detoxification by catalase (CAT). In addition, Corpas et al. (2001) reported that peroxisomes might be one of the cellular sites for nitric oxide (NO) biosynthesis. However, the role of NO in ROS metabolism in plants is still not known. $^1O_2$ production in plant cells was in the range of 240 µmol s^{-1} and a steady state level of H_2O_2 was in the range of 0.4 to 0.5 µM and photooxidative stress to the plant enhances the $^1O_2$ production to the range of 240-720 µM s^{-1} and a steady state H_2O_2 level of 5-15 µM (Mittler, 2002). Different sites of electron leakage and release of $O_2^{?-}$ and H_2O_2 from mitochondria have been reported (Tiwari et al., 2002). A site-specific release of free radicals has been associated with the activity of cyanide-insensitive alternative oxidase (McKersie and Leshem, 1994). In recent years, new sources of ROS have been identified including NADPH oxidases, amine oxidases and cell wall- bound peroxidases (Gross, 1980, Vianello and Marci, 1991, Dat et al., 2000). The generation of ROS is usually low under normal growth conditions. However stressful conditions including high light, drought, desiccation, salinity, low temperature, heat shock, heavy metals, UV- radiation, nutrient deprivation, pathogen attack and air pollution are known to disrupt cellular homeostasis through enhanced production of ROS (Bowler, 1992; Allen, 1995; Allen et al., 1997; Mittler, 2002; Luna et al., 2005). Increased generation of ROS is known to cause damage to the photosynthetic system as well as to other cellular components as shown in table 1.

Among these OH^-, being exclusively reactive, interacts with and damages several molecular species in plant cell (Zhang, 2003). $^1O_2$ and $O_2^{?-}$ predominantly attack chlorophylls and unsaturated fatty acids of cell and organelle membranes. D1-D2 proteins, Calvin cycle enzymes, Fe^{+2}-containing enzymes and Mn clusters in PS II are reported to be the targets of H_2O_2 (Havaux and Niyogi, 1999; Niyogi, 1999). In situations where $^1O_2$ formation rate exceeds the quenching capacity of the plant cell, increased $^1O_2$ can migrate outside the chloroplast and affect the unsaturated lipid components. Most recently, Rontani et al. (2005) reported $^1O_2$-mediated photooxidation of 18-hydroxy-oleic acid yielding 9-hydroperoxy-18-hydroxyoctadec 10(trans)enoic and 10-hydroperoxy-8-hydroxyoctadec 8-(trans)enoic-acids. These findings are significant as they clearly indicate the role of $^1O_2$ in the photooxidation of the unsaturated of higher plant lipid components.

5. DEFENSE SYSTEMS AGAINST PHOTOOXIDATIVE STRESS

Photoprotection in plants is a multi-component process in plants to overcome the potential damage arising from the absorption of excess light energy. This involves the balancing measure between the absorbed light energy and its utilization. The inevitable generation of ROS is due to the imbalance between these two processes. There are

Table 1. *Localization, half-life and target sites of different ROS in plant cells (Mittler , 2002; Perl-Treves and Perl, 2002)*

ROS	LOCALIZATION	HALF-LIFE	TARGET SITE
Dioxygen	Chloroplasts, Mitochondria	>100	Possibly not clear
Singlet oxygen	Chloroplasts	1×10^{-6}	Chlorophyll destruction, membrane lipid peroxidation
Superoxide radical	Chloroplasts, Mitochondria, Plasma membrane, Peroxisomes, Cell wall, Endoplasmic reticulum, Glyoxysomes	1×10^{-6}	Chlorophyll destruction, membrane lipid peroxidation, D1 protein
Hydrogen peroxide	Peroxisomes, Apoplast, Cell wall	Not known	Calvin cycle enzymes, cross linking to D1-D2, damage to Mn-cluster in PSII
Hydroxy radical	Chloroplasts, Cell wall	1×10^{-9}	All loci in cell

several strategies in plants for mitigation of photoinhibition which primarily involve the removal or detoxification of reactive oxygen molecules inevitably generated during photosynthesis.

5.1. Non-Enzymatic Antioxidants- Role of Plant Pigments

5.1.1. Pigments

The generation of $^1O_2$ under high light stress and other stressful conditions is highly deleterious to plant cell if it is not instantly removed. The toxicity of ROS arises from their ability to initiate radical cascade reactions that lead to protein damage, lipid peroxidation, DNA damage and finally cell death. Plants have evolved a range of avoidance and tolerance strategies employing versatile tools against photooxidative stress.

The use of solar energy in photosynthesis primarily depends on the ability to safely dissipate excess light energy to avoid photoinhibition. The dissipation process employed by plants in their natural environment is mediated by different groups of plant pigments which are known as photoprotective pigments. Carotenoids play an important role in the photoprotection of plant cell against over excitation in excess light and thus dissipate the excess of absorbed energy (Frank, 1999; Strzalka et al., 2003; Edreva, 2005a). Even under low light, carotenoids act as energetic antenna, harvesting light at the wavelength not absorbed by chloroplast and transferring electron excitation states towards photochemical reaction centers. Carotenoids are now known as intrinsic components of the chloroplast, involved in quenching the $^1O_2$ under excess light (Mittler, 2002). This quenching ability of the carotenoids was attributed to chain of isoprenic residues with numerous conjugated double bonds with delocalized Đ-electrons which allows easy energy uptake from excited molecules and dissipation of excess energy as heat (Edge et al., 1997; Edreva, 2005b). Also, â-carotein, lutein and neoxanthine are known to protect the photosynthetic apparatus against photoexcitation damage by quenching the triplet states of chlorophyll molecules (Frank, 1999). Carotenoids are thus potent scavengers of ROS, protecting pigments and lipids from oxidative damage (Edge and Truscott, 1999). Carotenoids also protect plants from photooxidative stress by modulating physical properties of photsynthetic membranes with an involvement of xanthophyll cycle (Demings-Adams and Adams, 1996).The quenching by exchange electron transfer to produce the carotenoid triplet state (^{3}Car) is the principle mechanism of carotenoid photoprotection against $^1O_{2.}$ Carotenoids fluidize the membrane in its gel state and make it more rigid in its liquid crystalline state. Changes in the membrane fluidity play an important regulatory role in the de-epoxidation of violaxanthine to antheraxanthine which influences the rate of xanthophyll cycle under high light stress (Havaux and Niyogi, 1999; Strzalka et al., 2003) (Figure 3).

Under excess light, a rapid change in the carotenoid composition of LHCs is a common phenomenon. The diepoxide xanthophyll violaxanthin is rapidly and reversibly converted to epoxide- free zeaxanthin via the intermediate antheraxanthin by the activity of violaxanthin deepoxidase and the reverse reaction is mediated by zeaxanthin epoxidase under low light regimes (Havauex and Niyogi, 1999). Zeaxanthin is known to quench the singlet excited states of chlorophylls or could favour protein – induced aggregation of the LHCs of PSII leading to energy dissipation, thus protecting the reaction centers from overexcitation and photoinhibition. Chloroplast membranes are sensitive targets for photodestruction by different ROS. The xanthophylls cycle is thus significant in scavenging the free radicals that otherwise would interact with the lipids surrounding the photosystems.The higher number of conjugated double bonds in antheraxanthin and zeaxanthin can be presumed to be better protectors than violaxanthin with a higher efficiency for deexciting $^1O_2$. The xanthophylls cycle is thus a ubiquitous light-controlled antioxidant system in which a simple chemical substitution in xanthophylls molecule elicits profound changes in the photostability of the chloroplast membrane system.

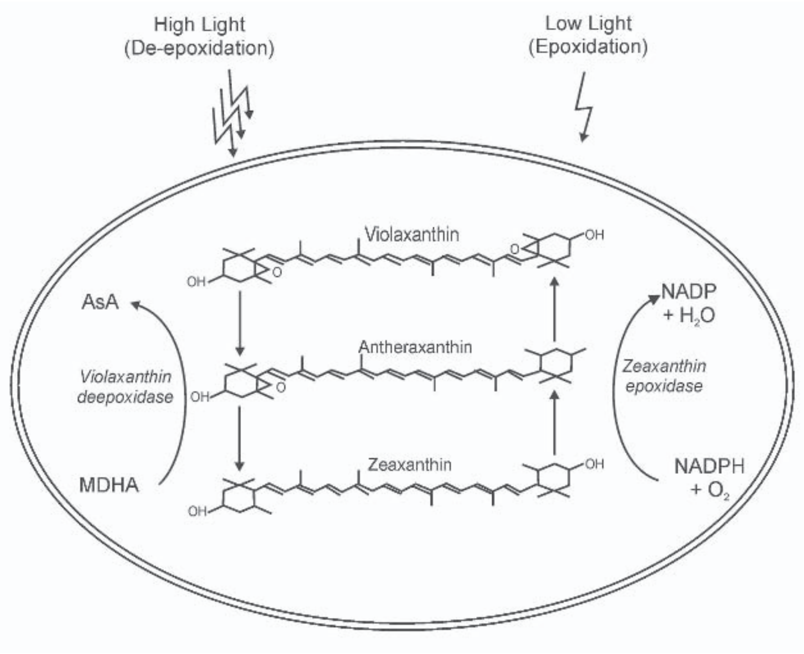

__Figure 3.__ Photo-regulation of xanthophyll cycle in plant cells

In addition, the light-regulated interconversion of photoprotective pigments like carotenoids confer a selective advantage under natural environment characterized by rapid changes in growth light intensity associated with other environmental constraints. Sun-acclimated leaves showed rapid increase in xanthophyll cycle-dependent energy dissipation compared to shade leaves. The sun leaves typically exhibited larger pool sizes of xanthophyll cycle pigments as well as their greater ability to convert this pool to antheraxanthine and zeaxanthine rapidly under high light (Bjorkman and Demmig-Adams, 1994). A large group of non-photosynthetic pigments including flavonoids (C_6-C_3-C_6 types) and the closely related anthocyanins (flavylium C_6-C_3-C_6^+ types) and betacyanins which are known for their screening out incoming visible and UV-radiations are reported to dissipate excess photon energy (Torel et al., 1986; Yutang et al., 1990; Winkel-Shirley, 2002; Edreva, 2005b). The antioxidant and ROS-scavenging ability of these non-photosynthetic pigments can protect the plant from photooxidative stress.

5.1.2. Ascorbic Acid (AsA)

L-Ascorbic acid (AsA) is an abundant metabolite in plant cells, some times reaching levels upto 10% of plant cell carbohydrate content (Smirnoff and Pallanca, 1995). AsA plays an important role in stress physiology of plants as well as plant growth and development. One of the most important activity of AsA is protection of plant cell against photooxidative stress (Davey et al., 2002). The biosynthesis of AsA and its involvement in protection against photooxidative stress suggest link between photosynthesis, light and AsA pool size. Leaf AsA content was markedly correlated with light intensity at the leaf surface (Foyer, 1993), Barley and *Arabidopsis* leaves accumulate significantly more AsA under high light compared to low light conditions (Conklin et al., 1997) of late, there is an increasing body of evidence confirming the role of AsA in the detoxification of ROS in the plants. AsA as the capacity to directly eliminate several different ROS, including $^1O_2$, O_2^-, $OH^?$ (Padh, 1990). AsA is also known to maintain the membrane bound antioxidant á-tocopherol in the reduced state (Liebler et al., 1986). In addition AsA plays a major role in photoprotection as a cofactor in the xanthophyll cycle (Conklin, 2001). The conversion of violaxanthin to zeaxanthin across the thylakoid membrane is thought to be involved in non-photochemical quenching of excess light energy in PSII (Deming-Adams and Adams, 1996). AA was shown to be required as a cofactor for the enzyme violoxanthine-de-epoxidase (Hager, 1969). AsA-deficient *Arabidopsis* mutants had lower levels of non-photochemical quenching due to a decrease in this de-epoxidation reaction (Conklin, 2001). However, the regeneration of AA in the plant cells is always associated with glutathione cycle as discussed below.

5.1.3. Glutathione

The non-protein, water-soluble and low molecular weight tripeptide thiol glutathione (GSH; ã-glutamyl cysteinyl glycine) plays a pivotal role in minimizing cellular disfunction, arising through stress induced redox perturbation (Vernoux et al., 2002). Interest in the benefits of genetically engineered cellular GSH concentrations in higher plants was prompted by the observations that elevated GSH levels correlated with stress tolerance. Successive oxidation and reduction of ascorbate, glutathione and NADPH would perform the potential scavenging of H_2O_2 generated through photooxidative stress in the chloroplast. These reactions are collectively referred as ascorbate-glutathione cycle (Figure 4). Later this pathway has been identified in other sub-cellular compartments including mitochondria, peroxisomes as well as in roots, endosperm, root nodules and petals (Bielawski and Jay, 1986; Klapheek et al., 1990; Mullineaux et al., 1996). Glutathione content in the plant cells is now used as a marker of oxidative stress in higher plants (Grill et al., 2001; Sopory, 2003; Tausz et al., 2004). Glutathione has also been shown as an antioxidant in mitochondria, cytosol, peroxisomes and nucleus (Noctor et al., 2002; Muller et al., 2002).

The ascorbate-glutathione cycle plays a crucial role in scavenging superoxide and H_2O_2 in plant cells. Reduced glutathione (GSH) acts as an electron donor to regenerate ascorbate from its oxidized form, dehydroascorbate. The redox ratio of dehydroascorbate/ ascorbate and GSH/GSSG are significant for the optimized operation of ascorbate glutathione cycle under conditions of photooxidative stress. Masi et al. (2002) reported increased turnover rates of glutathione in UV stressed maize leaves. GSH/GSSG ratios were higher in stress adapted plants indicating strong activation of ascorbate-glutathione cycle under photooxidative stress. However, Tausz (2004) postulates that the response of glutathione system to photooxidative stress will be differ-

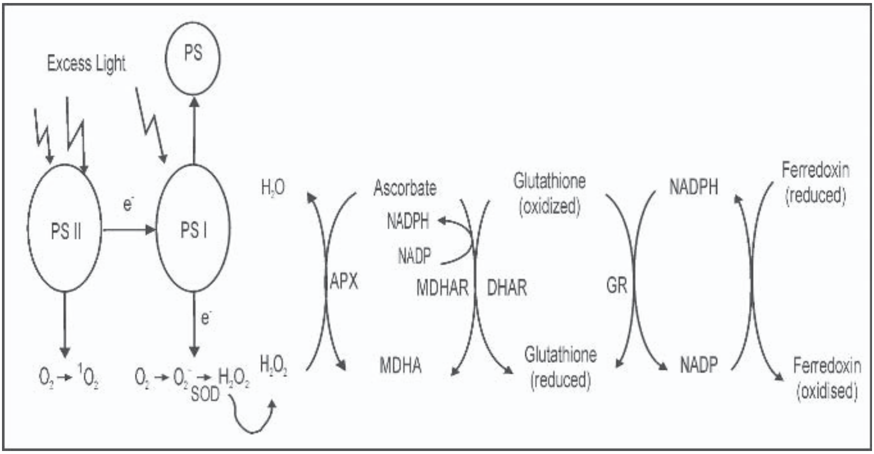

Figure 4 *. Ascorbate-Glutathione cycle as a defense system under photooxidative stress*

ential depending upon the environmental stress. In the initial reaction phase there may be a transient shift of the GSH/GSSG redox state towards slightly more oxidized value, while in an acclimation reaction, ROS triggers the production of glutathione by enhancing the enzyme activities associated with glutathione biosynthesis thus increasing the scavenging capacity of ascorbate-glutathione cycle (Baena-Gonzalez et al., 2001; Noctor et al., 2002; Neill et al., 2003). However, if the acclimatory responses of plants are too slow or too weak, photooxidative stress gradually depletes the glutathione content and the balance between oxidative load and the scavenging will be disproportionate, which leads to progressive oxidation and degradation of ascorbate and glutathione pools, eventually leading to senescence and plant death. Increased activities of ascorbate-glutathione cycle increased resistance to chilling related photooxidative stress in cot-

ton (Payton et al., 2001). Drought-induced photooxidative stress caused an oxidation of the glutathione pool in barley leaves (Smirnoff, 1993). It is imperative to believe that the tissue concentration of glutathione, redox states and glutathione-related enzymes confirm the central role of glutathione metabolism in plant responses to photooxidative stress. Further one might reasonably expect that the *in vivo* concentrations of glutathione influence the plant antioxidant capacity, the steady state levels and turnover of AsA.

5.1.4. Other Compounds as Antioxidants

Like carotenoids, tocopherols (vitamin E) are also known to involve in energy uptake and dissipation, a property determined by the presence of ring-closed conjugated double bonds in the benzene moiety of their molecule (Burton et al., 1982; Fryer, 1992; Halliwell and Guttiridge, 1999). Sugar alcohols such as mannitol can also serve as more efficient carbon sink for light reaction products and may therefore alleviate photooxidative stress (Smirnoff and Cumbus, 1989)

5.2. Enzymatic ROS Scavenging System in Plants

The ROS scavenging enzymes are located in different compartments of plant cells as already indicated in Figure 2. The ROS scavenging systems in plant cells consist of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDAR), dehydroascorbate reductase (DHAR), glutathione peroxidase (GPX) and glutathione reductase (GR) (Foyer, 2002; Reddy et al., 2004).

5.2.1. Superoxide Dismutase (SOD)

The SOD is one of the fastest enzymes (Vmax = 2×10^9 $M^{-1}s^{-1}$) with an optimum close to the diffusion rate of $O_2^{?-}$ (McCord and Fridovich, 1969). The conversion of $O_2^{?-}$ to H_2O_2 is the first link in the enzymatic scavenging of ROS and SOD is considered as the primary defense against oxygen radicals. Three different types of SOD have been found in plants containing either Mn, Fe or Cu and Zn as prosthetic metals (Asada, 1999). Different isoforms of SOD in plants are differentially expressed and localized in different compartments in the plant cell (Bowler et al., 1994; Wingle and Karpinski, 1996; Schinkel et al., 1998). Accordingly plant SODs are classified as MnSODs, FeSODs and Cu/ZnSODs and distinct isozymes have been identified in the cytosol, mitochondria and chloroplast (Bowler et al., 1994). Plant SODs are also reported in peroxisomes (Scandalios, 1997) and glyoxysomes (Bueno and del Rio, 1992). All plant SODs are encoded by the nuclear genome and organelle isozymes are transported post translationally to the appropriate cellular compartment (Perl-Treves et al., 1990). In maize, ten different SOD isozymes have been reported; four cytosolic Cu/Zn SODs,

four mitochondrial MnSODs and a novel type of chloroplastic FeSOD (Zhu and Scandalios, 1993; Kenodle and Scandalios, 1996). MnSOD, a homotetrameric mitochondrial enzyme of 85 kD has been isolated from *Nicotiana*, Pea, *Arabidopsis*, Rubber tree, Wheat and Rice (Breusegem et al., 2002).

5.2.2. Ascorbate Peroxidase (APX)

The APX in plant cells destroy harmful H_2O_2 via ascorbate-glutathione pathway (Figure 3), ascorbate-glutathione cycle provides protection against oxidative stress by a series of coupled redox reactions in photosynthetic tissues (Foyer and Halliwell, 1976), in mitochondria and peroxisomes (Jimenez et al., 1997). Based on the available sequence data, seven different APXs are distinguished in plants; two soluble cytosolic forms, three types of cytosol membrane bound, including a glyoxysome bound form, one chloroplastic stromal and one thylakoid membrane-bound (Jespersen et al., 1997). Among these, chloroplastic isoforms are very specific for ascorbate as electron donor while cytosolic APX transcript levels are induced mostly by drought and excess light (Vansuyt et al., 1997). Although APX activity was demonstrated in mitichondria and peroxisomes, intact mitochondria and peroxisomes had no latent APX activity indicating that the active site of APX is exposed to the cytosol and scavenges H_2O_2 leaking from mitochondria and microbodies (Jimenez et al., 1997). Glyoxysomal membranes of pumpkin, cotton and spinach were found to contain APX activity (Zhang et al., 1998; Yamaguchi et al., 1995). Recently Yabuta et al (2002) suggested that thylakoid APX is a limiting factor of ROS scavenging systems. Under photooxidative stress in chloroplast and that the enhanced activity of thylakoid APX functions to maintain the AsA content in the redox status of AsA under photooxidative stress.

5.2.3. Glutathione Reductase (GR)

The GR completes "Asada-Halliwell pathway" by regenerating glutathione pool with NADPH as electron donor (Foyer et al., 1994). Although most of GR activities were studied in chloroplasts, mitochondrial in cytosolic isoenzymes were also described in plant cells (Creissen et al., 1996). It was also reported that GR and glyoxalase II reduce GSSG to GSH and help in maintaining the plant cell in its homeostatic GSH/GSSG ratio (Kumar et al., 2003).

5.2.4. Glutathione-S-Transferase (GST)

The GST is another important enzyme which protects the plant cells by detoxifying harmful compounds. The GST is known to catalyze the conjugation of GSH to a variety of cytotoxic compounds (Kumar et al., 2003). Glutathione transferases (GSTs) are also known to play important role in protecting the plants from wide range of biotic and abiotic stresses including xenobiotic toxins, UV-radiations and photooxidative stress

(Zeng et al., 2005). GSTs are also induced in the transport of toxic secondary products and cell signaling during stress responses (Agarwal et al., 2002; Loyall et al., 2002). Also, free radical-induced toxic compounds including lipid peroxidases (4-hydroxy-alkenals) and products of oxidative DNA damage are congjugated to GSH by GST and detoxified or sequestered into vacuole for further degradation (Coleman et al., 1997). The GST is thus considered as a key enzyme to maintain glutathione homeostasis.

5.2.5. Catalase (CAT)

Catalase (CAT) effectively scavenges H_2O_2. In plants tissues, CAT is localized in peroxisomes to scavenge H_2O_2 produced by glycolate oxidase in photorespiratory cycle. The activities of CAT were also reported from mitochondria in plants but not in chloroplasts (Scandalios et al., 1980). Transgenic tobacco plants, deficient in CAT isozymes, developed necrotic lesions under high light which indicated that CAT is necessary for protection of plant cells against photooxidative stress (Chamnogpol et al., 1998). Catalase protein synthesis has been linked to photosynthetic and photorespiratory pathways (Schmidt et al., 2002).

5.2.6. Antioxidant Enzymes in C_4 Plants

Antioxidant enzymes are known to be distributed among all photosynthetic cells in higher plants. The distribution of antioxidant enzymes between mesophyll and bundle sheath cells in C_4 plants have been recently reported (Foyer, 2002, Sundar et al., 2004). GR and DHAR were exclusively localized in mesophyll cells whereas most of the SOD and APX were reported in the extracts from both mesophyll and bundle sheath cells. CAT and MDHR were approximately equally distributed between mesophyll and bundle sheath tissues. H_2O_2 was found to accumulate only in mesophyll cells. The localization studies on the antioxidant enzymes in C_4 plants are very interesting because the enzymes of PCR cycle, which are sensitive to H_2O_2 are located safely in bundle sheath cells. Kingston-Smith and Foyer (2000) suggested that oxidative damage under stressful conditions, if any, in C_4 plants will be restricted to bundle sheath tissue because of inadequate antioxidant protection in this tissue. However, very little mechanistic information is available on photooxidative stress-induced antioxidative metabolism between the two cell types in C_4 plants.

5.2.7. Other Scavenging Proteins

Thioredoxin is a small ubiquitous protein that plays a redox-regulatory role in plants. H_2O_2 is a potent oxidant of enzymes thiol groups and its inhibitory effects on CO_2 fixation is due to the inactivation of thiol regulated enzymes. In illuminated chloroplasts, an electron flux may occur via the thioredoxin system (Polle, 1997). Several stromal enzymes are light regulated via this system, which transfers reducing equiva-

lents from PSI through ferridoxin-thioredoxin reductase (Buchanan, 1991; Zhang and Portis, 1999). Due to high generation of ROS in light, continuous reduction of target enzymes is necessary to maintain active sites for all further reactions. Thiol modulation of proteins in chloroplast metabolism to regulate the flux of electrons through electron transport complex is now well characterized (Noctor et al., 2004). Electron transfer from thioredoxin and glutathione peroxidases to peroxides has also been reported (Baier and Dietz, 1999; Mullineaux et al., 1998). Plant cell cytoplasm depends on a reduced states of its thiol-/disulfide system with redox potentials usually ranging from -240 to -300 mV to counteract oxidative damage (Baier and Dietz, 1999). Peroxiredoxins (Prx) constitute essential elements in detoxifying ROS under high light conditions of photosynthesis. All Prx have cysteinyl residue to catalyze the reduction of various peroxide substrates ranging from H_2O_2, lipid peroxides to peroxinitrite and the palnt Prx are grouped in four distinct classes, the 1-Cys Prx, the 2-Cys Prx, the Prx Q and the type II Prx (Hofmann et al., 2002). Dietz et al (2004) suggest that Prx are abundant and essential elements of the redox homeostatic networks of chloroplasts and specific interactions within the redox network of the chloroplast need to be addressed in addition to a detailed study of the effect of Prx on photosynthesis to understand the function of different Prxs in antioxidant defense and redox signaling in plant cells.

6. PLANT ACCLIMATION TO PHOTOOXIDATIVE STRESS

The activity of plants to adapt to changing light environment allows them to achieve great evolutionary success exemplified by growing in contrasting habitats of high irradiation intensity. These variations in light environment would be on a time scale ranging from few seconds and minutes up to few and even many days. Long-term responses to irradiation variations may be elicited at the whole plant, leaf and chloroplast level (Bailey et al., 2001). Plants acclimated to high irradiance use several mechanisms to protect the photosynthetic apparatus against deleterious effects of photooxidative stress. Much attention on acclimation to high irradiance has been focused on xanthophyll cycle in the dissipation of excess excitation energy in the light harvesting antennae (Demmig-Adams and Adams, 1996). Xanthophyll cycle-dependent energy dissipation lowers the photon efficiency of PSII and provides a mechanism to balance the synthesis of ATP and NADPH with the rate at which these metabolites can be utilized in photosynthesis (Noctor and Foyer, 2000). Some studies directly addressed whether growth at high irradiance induces an increase in cellular antioxidant systems due to higher rates of O_2 photoreduction (Grace and Logan, 1996; Niyogi, 1999; Osmond et al., 1999; Matsubara et al., 2004). Longer-term acclimation to high light appeared to have a positive effect on antioxidant systems. Logan et al (1996) reported that with increasing levels of irradiance, there was a concomitant increase in ascorbate and xanthophyll cycle pools in plants, which provide photoprotection.

It is well known that high light acclimated plants had less number of chlorophyll a/b binding light harvesting complexes of PSII (LHC II) per PSII reaction center

and this reduction of LHC II in response to high light was partially attributed to acclimative proteolysis of apoproteins of outer LHC II (Anderson et al., 1995; Boekema et al., 1999; Jackowski et al., 2003). However, the biochemical identity of products of LHC-gene family and their possible role as irradiance-responsive protein is yet to be understood. Jackowski et al. (2003) demonstrated that plant acclimation to high light irradiance was accompanied by progressive decline in Lhcb 2 and 3 abundance, whereas decline in Lhcb1 level was identified only at excessive irradiance causing moderate stress to PSII and there was an acclimation related decline in LHC II apoproteins in spinach. Ascorbate exhibited the most dramatic acclimatory response to growth photosynthetic photon flux density among all antioxidants (Grace and Logan, 1996). This can be attributed to multi-faceted roles of ascorbic acid in plant cell metabolism, particularly in photoprotection of the chloroplast. In addition, it is also believed that the redox signals derived from photosynthetic electron transport play an important regulatory role in acclimation to high light stress. The redox signals are known to modulate the expression of many plastid and nuclear genes encoding photosystems (Walters, 2004). Plant acclimation to high light results in an increase in the photosynthetic rate which has the potential benefit to the plant as increased photosynthetic rates increase the growth rates. In contrast, antisense plants with reduced levels of cyt *bf* complex have marked reductions in net photosynthetic rates indicating that large changes in the levels of cyt *bf* complex under low light are intimately linked to the changes in photosynthetic capacity (Price et al., 1998). Also, high light-grown plants often have substantially increased capacities for Δ pH-dependent protective energy dissipation (qE), which were related to different energy dissipation characteristics of a larger light harvesting system (Park et al., 1996; Bailey et al., 2004). These studies suggest that redox regulation and antioxidant systems in plant cells appear to be part of acclimatory responses of plants to high growth light intensities. Furthermore, mutants defective in acclimation to photooxidative stress will be critical tools to understand the adaptive benefits to photoacclimation. It would be possible to consider the ways in which modifying acclimation behavior of plants might help to improve the agriculture productivity in crop plants under changing global environmental conditions.

7. MOLECULAR AND GENETIC ASPECTS OF PLANT RESPONSES TO PHOTOOXIDATIVE STRESS

Light is highly unpredictable resource for plants and the changes in growth irradiance induce several changes in biochemical and molecular composition of the plant cell. Murchie et al. (2005) showed that there are 99-light responsive genes which were down regulated and 130 were up-regulated in rice during light treatment. Majority of these genes showed reduced levels of expression in response to high light, whereas stress related genes showed increased level of expression. In order to avoid over-excitation of chlorophyll protein complexes and photooxidation, a regulated degradation of LHC was observed in rice leaves along with a decline in CP-24, PSI genes and a 10 kD PSII

gene was also noticed under high light (Murchie et al., 2005). PS I has long been reported to be less affected than PSII by high light (Kok, 1956). PSI in isolated thylakoid membranes was inactivated by high light (Sonoike, 1995). Since PSI is the terminal electron carrier in the chloroplast, it was identified as a major site producing ROS and shown to be closely associated with ROS-scavenging systems in the chloroplast (Ogawa et al., 1995). The role of ROS inactivating PSI reaction center and degradation of psaA and psaB under high light conditions has been studied (Sonoike, 1996; Tjus et al., 1999). Very recently, Jiao et al (2004) demonstrated that high light stress readily photoinhibited PSI, following the loss of psaC as well as degradation of PSI reaction center proteins (psaA and psaB). The findings suggest that PSI photoinhibition can be a limiting factor in crop productivity under high light.

Photoinhibition and photooxidative stress reflect the photoinactivation of photosynthetic apparatus especially the PSII and thus decreasing the photosynthetic function which is often referred to PSII photoinhibition and degradation of D1 proteins (Long and Humphries, 1994; Kettuhen et al., 1996). Damaged PSII centers do not usually accumulate in the thylakoid membrane due to a rapid and efficient repair mechanism .As most of the damage is targeted to D1 protein, the turnover and repair of the protein subunits is a complex process involving reversible phosphorylation of PSII core subunits, monomerization and migration of PSII core, highly specific proteolysis of the damaged proteins and multi-step replacement of the damaged proteins with *de novo* synthesized copies (Aro et al., 2004). In addition to D1 protein, it was also reported that D2 protein also occasionally becomes damaged in light (Sansen et al., 1996). More recently, one of the small PSII subunits, the psbH protein was also shown to be frequently replaced (Bergantino et al., 2003). Although phosphorylation of all the major LHCII antenna proteins are not involved in PSII photodamage or repair, the phosphorylation of Lhcb4 (CP29) has been assigned a role in the photoprotection of PSII centers and this protein is a relevant candidate to possess a functional role in the dissipation of excess excitation energy and the protection of PSII against photodamage (Bassi et al., 1997; Bergantino et al., 1998; Pursiheimo et al., 2003). On the other hand, another PSII protein in thylakoids, the 22 kD psbS protein is now known to function in the regulation of photosynthetic light harvesting and is necessary for photoprotective thermal dissipation (qE) of excess absorbed light energy in plants (Niyogi et al., 2004; Hieber et al., 2004). *Arabidopsis thaliana* mutants lacking qE required psbS in addition to low lumen, pH and the presence of de-epoxidized xanthophylls for protoprotection (Li et al., 2002).

The expression of LHC genes is tightly regulated by light (Niyogi, 1999). High light intensities inhibit transcription of LHC genes and activate a set of proteins known as early light-induced proteins (ELIPs), a class of proteins structurally related to LHCs (Hutin et al., 2003). ELIPs are predicted to have three trans-membrane helices like LHCs and are known to bind chlorophyll and carotenoids. Recently, a number of ELIP-type polypeptides in response to high light have been discovered in higher plants (Jasson et al., 2000). The induction of ELIPs in plants by high light intensities suggests a role in the acclimation to light stress rather than a light harvesting function. ELIPs are also

known to protect plant leaves from photooxidation and suggest that this photoprotective function involves the maintenance of a low level of free chlorophyll under high light conditions to minimize the formation of $^1O_2$. ELIPs are thus known as scavengers of free chlorophyll molecules released during the rapid turnover of the photosynthetic complexes and the reorganization of photosynthetic machinery in high light (Adamska, 1997; Hutin et al., 2003). ELIPs are also considered as plant defense systems in light stress conditions, which have the potential to become new selection markers for the identification and development of transgenic crop plants, tolerant to photooxidative stress conditions.

The APX is a regulatory enzyme in ascorbate-glutathione cycle. Furthermore, this cycle is essential for the continuous regeneration of ascorbate and glutathione which are known to be rapidly depleted under photooxidative stress. Recently, it was demonstrated that chloroplastic APXs (chl APXs) were inactivated as a result of the change of redox status of AsA under photooxidative stress (Yoshimura, 2000). Chl APXs were more strongly inactivated than thiol-modulated enzymes in the Calvin cycle, which are believed to be the most sensitive enzymes to H_2O_2. Yabuta et al. (2002) demonstrated that transgenic tobacco plants (TpTAP-12) overexpressing APX (37-fold high activity than the wild-type plants) showed increased tolerance to photooxidative stress as well as to chilling stress with high light intensity. Nevertheless, photooxidative tress may not have detrimental effects if scavenging of ROS is triggered in the proper cell compartment. Targeting of APX and SOD to chloroplasts resulted in increased stress tolerance in tobacco to high light (Kwon et al., 2002; Yabuta et al., 2002). Chloroplastic overexpression of GR can increase the leaf GSH/GSSG ratio and mitigate the damage due to photooxidative stress (Foyer and Noctor, 2001). Increased defense against photooxidative stress was also conferred by targeting bacterial CAT to tobacco chloroplasts (Mohamed et al., 2003). Induction of glutathione peroxidases and 2-cysteine peroxiredoxins was also suggested to be crucial in controlling chain type reaction that follows the initiation of lipid peroxidation by $^1O_2$ and $OH^?$ in plant cell membranes (Mullineaux et al., 1998; Bair and Dietz, 1999).

Transgenic plants have been produced which overaccumulated ROS-scavenging metabolites or overexpressed ROS-scavenging enzymes (SOD, CAT, APX, GR) for improved oxidative stress tolerance which also showed enhanced yield and survival under environmental stress conditions (Edreva, 2005a). Overexpression of SOD in alfalfa conferred tolerance to high light (Alscher et al., 2002). Miyagawa et al (2000) showed that CAT from *E.coli* with higher affinity for H_2O_2 than plant CATs, was overexpressed in tobacco thus conferring protection to high light stress. A deeper understanding of such submolecular bases of ROS-related processes may constitute a rationale for developing transgenic plants for tolerance to photooxidative stress. Murchie et al. (2005) showed an up-regulation of genes involved photoprotection and photooxidative stress when rice plants were treated with high irradiance. A significant increase in the level of expression of MDHAR was observed. High light intensities also up-regulated the activities of APX (Karpinski et al., 1999; Rossel et al., 2002). In *Arabidopsis*,

β-carotene hydroxylase gene was up-regulated by high light which is involved in xanthophylls-cycle carotenoid biosynthesis with increased leaf contents of xanthophylls and higher tolerance to high light and heat stress (Davison et al., 2002). Rossel et al. (2002) also concluded that photooxidative stress-induced genes also trigger the production of cold and heat shock proteins providing a comprehensive tolerance mechanism to the plant against unfavourable environmental variables.

Very interestingly, transgenic rice plants expressing C_4 photosynthetic enzymes have shown significantly high tolerance to photooxidation compared to wild-type rice (Jiao et al., 2002). Earlier Jiao and Ji (1996) showed that transgenic rice with PEP carboxylase may play an important role in adaptation to photooxidative stress. PSII electron transport efficiency as determined by Chl a fluorescence analysis (Fv/Fm) of transgenic rice with C_4 enzymes (PEPC, PEPCK and ADPME) was high compared to wild-type rice after photooxidative stress treatment (Jiao et al., 2002). Consistently, the ability to dissipate the excess light energy by photochemical and non-photochemical quenching increased more after photooxidative stress treatment. The transgenic rice plants also showed higher light intensity for saturation of photosynthesis, high photosynthetic CO_2 uptake rates and significantly produced 24% more grains than wild-type plants. These findings suggest that expression of C_4 photosynthetic enzymes in C_3 plants can certainly improve tolerance to photooxidative capacity. However, the exact mechanism responsible for this improved tolerance to photooxidative stress in transgenic C_3 plants remains to be elucidated. The induction of genes involved in protection against photooxidative stress suggests the presence of increased amounts of ROS, which are also known to be involved in cell signaling processes for cross-tolerance.

8. ROS AS SECONDARY MESSENGERS

Plants are continually in danger of absorbing more light energy than they can use for their metabolism. The excess excitation energy has to be dissipated to avoid photooxidative damage to the photosynthetic apparatus, which is often manifested as leaf bleaching, chlorosis or bronzing of leaves (Niyogi, 2000). Immediate responses to the conditions that promote excess excitation energy would initiate signaling pathways for plant acclimation. In response to excess light energy absorption, there would be increases in the rates of electron transport and consequent redox changes in photosynthetic electron transport which in turn regulates the expression of both nuclear and chloroplast genes to encode components of photosynthesis and antioxidant metabolism. Examples of such genes are *Cab* (encodes chlorophyll a/b binding protein), *LHC* (gene for LHCl), *APX1* and *APX2* (Kripinski et al., 1999; Oswald et al., 2000). Redox changes in the vicinity of Q_A and Q_B or plastoquinone have been suggested to be key starting points for signaling (Mullineaux and Kripinski 2002).

The ROS have been ascribed signal functions both in biotic and abiotic stresses. As described above, high concentrations of ROS are extremely harmful to plants; while

in lower concentrations, ROS are involved in cell signaling, acclimation and cross-tolerance (Dat et al., 2000; Neill et al., 2002). Of all the ROS molecules, H_2O_2 is thought to freely diffuse across biological membranes (Kripinski et al., 1999). Chloroplast-derived H_2O_2 could directly influence the functions of extra-plastidial signaling components which play a potential role in the systemic responses of plants to excess light. H_2O_2 plays a major role in triggering the expression of antioxidant enzymes (SOD, CAT, APX, GR) for photoprotection (Gechev et al., 2002; Neill et al., 2002). H_2O_2 is relatively stable ROS, unchanged at physiological pH and move out of the sites of its generation propagating throughout the plant, specially interacting with components of redox-signaling pathways in plants (Noctor et al., 2002). When low light grown plants were irradiated with high light, a burst of H_2O_2 and photoinhibition occurred. H_2O_2 is known to act as intracellular signal coming out of the chloroplast into cytosol, inducing a second line of defense and counteracting the photoinhibition (Mulineaux and Karpinski, 2002). Hence H_2O_2 is considered as an intra and intercellular as well as interorgan systemic signal which is involved in the acclimation of plants to high growth irradiances. The apoplastic enzyme ascorbate oxidase also regulates the redox state of the apoplastic ascorbate pool (Pignocchi and Foyer., 2003). The function of ascorbate oxidase is to modify the apoplastic redox state in such a way as to modify receptor activity and signal transduction to regulate photooxidative stress. ROS-mediated signaling mechanisms described above depend on the passage of ROS molecules out of the chloroplast to propagate a signal leading to nuclear gene expression. It is thus believed that electron transport chain starts with NADPH in the stroma, spans the chloroplast envelope and ends with O_2 as the terminal electron acceptor on the outer surface of the chloroplast. Chloroplast envelope is known to contain several constituents including iron-sulphur proteins, semiquinones, flavins and α-tocopherol that are involved in the transfer of electrons and therefore could provide another exit for a chloroplast-derived signal (Jager-Vottero et al., 1997).

Recently, mitogen-actiavted protein (MAP) kinases are known to be involved in the signal transduction pathway of plants that senses ROS. At MAPK 3/6 and NL-p 46 MAPK and NtANP1, NtNPK1 have been implicated in ROS signaling in *Arabidopsis* and tobacco (Mittler, 2002). When H_2O_2 is sensed by sensors, calmodulin and MAP kinase cascade would be activated, resulting in activation or suppression of transcription factors, thus regulating the response of plants to oxidative stress. The involvement of ROS in the regulation of stomatal closure and auxin-related cellular responses also suggest that more signaling pathways might be involving ROS as inducers of systemic signals in plants under conditions of oxidative stress. The use of transgenic plants with a comprehensive analysis of ROS-producing and ROS-scavenging systems by using genomics and proteomics should unravel the further role of ROS in signal transduction in plants.

9. CONCLUDING REMARKS

Light is critical for growth and development of plants. It is apparent from the foregone account that plants have evolved multiple strategies of photoprotective and photoadaptive mechanisms that are critical for survival under conditions of excess photon absorption. Some of these mechanisms involve changes in pigment-protein complexes, synthesis and recruitment of enzymes with antioxidant function and abundant soluble antioxidants in the chloroplasts. Furthermore, the redox conditions in plant cells may modulate the photosystem components for acclimation to photooxidative stress. The induction of genes under photooxidative stress for optimized absorption of light by specific photoreceptors is poorly understood. Analysing the expression of these genes under different light quality/quantity conditions will enable us to make specific inferences about the nature and sensitivity of such photoreceptors in plants. Genetic regulation of antioxidant biosynthesis and overexpression several antioxidative enzymes should reveal plant acclamatory responses in response to photooxidative stress. There is a need to understand how the photooxidative stress provoke several changes in cellular metabolism, reflecting the changed expression of a common or overlapping set of genes. Detailed information is also required on how the signaling molecules are integrated with ROS into the general signaling network of a cell and/or the intracellular production sites affect the signaling pathway. Metabolite, protein and transcript profiling technology could provide us a holistic understanding of how plants thrive in highly variable and adverse environments.

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CHAPTER 7

NUTRIENT STRESS

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1. INTRODUCTION

Mineral nutrients are essential for normal growth and development of plants. The phenomenal growth of knowledge made in the areas of the mechanism of the ion uptake, the critical role of minerals in the basic processes at cellular level and molecular approaches to the study of mineral nutrition have raised the status of mineral nutrition of plants as an independent discipline of the plant biology (Epstein, 1972; Mengel and Kirkby, 1978; Clarkson and Hanson, 1980; Marschner, 1995; Loneragan, 1997; Grossman and Takahashi, 2001).

Chemical analysis of plants revealed the occurrence of about 60 elements in different tissues although only 17 elements are essential for growth and metabolism. These seventeen elements were classified into two groups depending on the concentration needed by the plants and they were designated as macro and micro nutrients. Ideally the classification should be based on biological structure and metabolic function. According to Mengel and Kirkby (1978) they were divided in to four groups depending on their chemical nature. The first group includes C, H, O, N and S, which in reduced form are covalently bonded constituents of the plant organic matter. The second group consists of P and B which occur as oxyanions, phosphate, borate and silicate. The third includes K, Na, Ca, Mg, Mn and Cl, which are associated with osmotic and ion balance roles. The fourth group of plant nutrients is made of Fe, Zn, Cu and Mo. These elements are present as structural chelates or metalloproteins.

The nutrient requirement of plants can be assessed roughly by inorganic composition of the plant. Plant dry matter constitutes 10-20 percent of the fresh weight. Nearly 10 percent of the dry matter consists of mineral elements. The mineral composition of plants show a lot of variation and is influenced by several factors such as genetic

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constitution of the plant, chemical constituents of the soil, climatic conditions and age of the plant. Despite wide variation in the mineral composition of different plants, a certain critical level of nutrients is necessary for healthy growth of plants. An idea of elemental composition with relative levels of each of the nutrients for higher plants is indicated in Table 1.

Table 1. *Concentration of mineral elements in soil, available form and their content in plants**

Element	*Available form*	*Content in soil*	*Content in dry matter (μmol/g)*
Molybdenum	MoO_4^{2-}	0.2 – 2.0 ppm	0.001
Nickel	Ni^{2+}	< 100 ppm	0.001
Copper	Cu^{+} & Cu^{2+}	5 – 50 ppm	0.10
Zinc	Zn^{2+}	10 – 30 ppm	0.30
Manganese	Mn^{2+}	200 – 300 ppm	1.00
Iron	Fe^{2+} & Fe^{3+}	2,50,000 ppm	2.00
Boron	BO_3	20 – 200 ppm	2.00
Chlorine	Cl^{-}		3.00
Sulphur	SO_4^{2-}	0.04%	30
Phosphorus	H_2PO^{4-}	0.1%	60
Magnesium	Mg^{2+}	0.05%	80
Calcium	Ca^{2+}	0.5%	125
Potassium	K^{+}	2.0%	250
Nitrogen	NO_3^{-} & NH_4^{+}		1000

**Adapted from Epstein (1965), Brown et al. (1987), Hopkins and Hüner (2004).*

Nutrient stress is a complex phenomenon, understanding of which requires the co-ordinated efforts of soil scientists, ecologists, physiologists, biochemists, agronomists and molecular biologists. Nutrient stress may result from either by low levels of availability of the element or by the presence of excess concentrations. In some cases the presence of one element in excess concentrations may induce the deficiency of another element. In this context, attempts were made to show the availability, functional aspects, and deficiency and toxicity symptoms of 14 elements essential for the survival of plants and other elements, which produce phytotoxicity. Visual deficiency symptoms provide a valuable basis for assessing the nutritional status of the plant. Deficiency symptoms are the consequence of metabolic disturbances at various stages

of plant growth. Nutrient deficiency symptoms in plants vary from species to species and from element to element. In general the symptoms are yellowing of leaves, darker than normal green colour, interveinal chlorosis, necrosis and twisting of leaves.

Toxic levels of metals in soils are attained by the metal bearing soluble constituents in the natural soil or by waste disposal practices in mining, industrial manufacture and urban sewage (Brown and Jones, 1975). Generally, each metal causing phytotoxicity would produce certain characteristic symptoms. The most general symptoms are stunting of growth and chlorosis of leaves. The toxicity symptoms observed may be due to a specific toxicity of the metal to the crop or due to an antagonism with other nutrients.

Plant analysis is an important diagnostic tool in assessing the nutritional disorders and in monitoring the nutrient levels. Soil and plant analysis by appropriate techniques like Atomic Absorption Spectrophotometry (AAS) and Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES) provide accurate levels of nutrients sufficient for plant growth and development. By comparing the analysis of both soil and plant tissues it is possible to assess the nutritional requirements of elements. Soil, environmental and management practices cause either deficient or in excess nutrient levels in plants.

2. NITROGEN

Nitrogen is one of the most prevalent elements and it is a component of amino acids, proteins, nucleic acids, chlorophyll and many other metabolites essential for survival of the plant. It is available to plants in four different forms viz., N_2, NO_3^-, NO_2^- and NH_4^+. Numerous field experiments conducted throughout the world has shown that nitrogen is the most important growth limiting factor. Nitrogen application is one of the important nutrient amendments made to the soil to improve growth and yield of many crop plants.

Deficiency of nitrogen profoundly influence the morphology and physiology of plants. Plants under low levels of nitrogen develop an elevated root : shoot ratio with shortened lateral branches. Higher levels of NO_3^- inhibits root growth and leads to a decrease in the root : shoot ratio (Scheible et al., 1997; Zhang et al., 1999). Under nitrogen deficiency, plants exhibits stunted growth and small leaves. In the beginning of nitrogen deficiency the older leaves show chlorosis when compared to younger leaves because of high mobility of nitrogen through phloem. Nitrogen deficiency induces the chloroplast disintegration and loss of chlorophyll. Necrosis occurs at later stages and if nitrogen deficiency continues it ultimately results in plant death.

When plants are provided with higher amounts of nitrogen they exhibit succulent growth, dark green leaves and the leaves become thick and brittle. Fruit set also will be hampered. Liu et al., (2000) found that nitrate may be involved in the biosynthesis of cytokinin or transport of cytokinins from roots to leaves. Several genes are associated with the induction of nitrate transporters (Forde, 2000).

World nitrogen consumption has increased from 22 to 80 million tons in the last 20 years. Anhydrous ammonia is the main ingredient of most of the nitrogenous fertilizers.

3. PHOSPHORUS

Phosphorus is the second important nutrient required by plants .It is an essential component of nucleic acids, phosphorylated sugars, lipids and proteins which control all life processes. Phosphorus forms high energy phosphate bonds with adenine, guanine and uridine which act as carriers of energy for many biological reactions.

Phosphorus is present in the soil in inorganic and organic forms. Much of the inorganic phosphorus is present mainly as $H_2PO_4^-$ and HPO_4^{2-}. Availability of these ions depend on soil pH. The lower pH favours $H_2PO_4^-$ and the higher pH HPO_4^{2-}. The main source of organic phosphorus is plant and animal debris residue and this is degraded by microorganisms to release in organic phosphorus. Vance et al (2003) found that plant growth is limited because of the inaccessible and unavailable form in the soil. Arbuscular mycorrhizae (AM fungi) promote the plant growth by the improved supply of phosphorus from the soil (Tinker et al., 1992). Since the phosphate availability is usually low in the soils, the plants have developed special adaptations to acquire the same with the help of multiple high affinity transporters (Raghothama, 1999).

The requirement of phosphorus for optimal growth is in the range of 0.3 to 0.5% of the plant dry matter. The toxicity may occur if the tissue concentration is more than 1% in the dry matter. Phosphorus deficiency decreased photosynthetic rate in soyabean leaves (Lauer et al., 1989). Root growth is less affected under phosphorus deficiency than shoot growth leading to a typical decrease in shoot –root dry weight ratio (Fredeen et al., 1989).Phosphorus deficiency not only retard shoot growth but also affects the formation of reproductive organs (Barry and Miller, 1989). Toyota et al., (2003) observed that phosphorus deficiency induced the expression of phosphoenolpyruvate carboxylase in Tobacco.

Phosphorus deficient plants show stunted growth, leaves develop characteristic dark blue green colour and some times purplish appearance. Because of the high mobility of phosphorus older leaves become chlorotic as compared to younger leaves. Leaf shape may be distorted and also leads to reduction in the number of leaves (Lynch *et al.,* 1991). Phosphorus deficiency caused decrease in primary root elongation and increased lateral root formation.(Lynch and Brown, 2001; Hodge, 2004). Phosphorus toxicity induces iron and zinc deficiency. At higher levels of P the yield was decreased in *Abelmoschus esculentus* L. due to the deficiency of zinc (Table 2).

Main source for phosphorus fertilizers is the rock phosphate. super phosphate and Diammonium phosphate are important phosphorus fertilizers. In response to phosphorus deficiency several genes are expresssed. Expression of these genes could be used to develop the crop plants for the improved phosphorus use efficiency. (Wang et al., 2002; Hammond et al., 2003; Franco-Zorrilla et al., 2004).

***Table 2.** Effect of phosphorus and zinc (Kg/ha) on dry matter yield of tops and roots and fruit yield in okra (Abelmoschus esculentus L. (v. Pusa sawani)**

	Dry matter yield (g)/plant								Fruit yield (q/ha)			
	Tops				Roots							
	Zn_0	Zn_5	Zn_{10}	Zn_{25}	Zn_0	Zn_5	Zn_{10}	Zn_{25}	Zn_0	Zn_5	Zn_{10}	Zn_{25}
P_0	2.06	2.30	1.86	1.20	1.08	1.92	1.34	0.60	44.30	62.16	56.47	40.62
P_{50}	2.88	2.18	2.58	1.62	1.52	1.24	1.48	0.75	89.20	55.80	68.65	54.62
P_{100}	3.16	3.68	4.01	1.85	1.81	2.00	2.20	0.88	106.1	109.0	121.1	58.84
P_{300}	1.25	2.61	2.04	1.40	0.68	0.95	0.83	0.66	39.51	59.77	56.05	50.18

* *Usha Kumari and Reddy (2000).*

4. POTASSIUM

Potassium (K^+) is a monovalent cation and usually occurs in its hydrated form. Potasium content in the earth's crust is around 2.3%. Soil K^+ can be divided in to three fractions

1. K^+ as a structural element of soil minerals.
2. K^+ adsorbed as exchangeable from soil to clay minerals and organic matter.
3. K^+ present in the soil solution.

Potassium plays a pivotal role in plant growth and development. Membrane K^+ channels are essential for the transport of K^+ between the cell compartments and cells with in a tissue. The channels open and close at different sequence and length in response to environmental signals and cause changes in the electro potential of the membrane and control entry of K^+. A high affinity transporter, HKT1 responsible for K^+ transport and K^+- Na^+ symport has been identified (Fox and Guerinot, 1998).

Potassium promotes cell elongation and maintains osmoregulation. Potassium promotes photosynthetic rate and controls the rate of transport of photosynthates from source to sink. Potassium is also essential for protein synthesis and activates nearly 45 enzymes involved in various metabolic processes.

Potassium is required in 2 to 5% of dry weight of plants. Potassium deficiency induces reduced growth, shortening of inter nodes followed by bushy appearance, chlorosis and necrosis. The symptoms first appear on older leaves. K^+ deficient plants are susceptible to lodging and drought (Lindhauer,1985). Abundant K^+ supply causes

nitrogen deficiency and may interfere with the uptake of divalent cations like Ca^{2+} and Mg^{2+}.

Potassium fertilization is necessary to improve the yield of almost all crops. In general K^+ application is about 50 to 250 kg /ha^{-1}year^{-1}. Potassium is mainly supplied as chemical fertilizer to crops in the form of "muriate of potash"(KCl) and other sources are K_2SO_4 and KNO_3. Potassium may not form structural organic compounds and yet is required in high quantities to perform various roles such as osmoregulation, enzyme activation, neutralization, transport processes (Table 3).

Table 3: *Effect of foliar application of potassium on yield components of two pigeonpea cultivars**

Yield component	*C V PDM$_1$*		*C V LRG30*	
	Control	*K^+*	*Control*	*K^+*
Leaf area (cm^2)	776	2504	757.5	1450
Number of pods/ plant	107	221	119	174
Number of seeds/Plant	428	884	486	647
Grain yield (kg/acre)	333	637	334	539

**Adapted from Ravindranath et al. (1985).*

5. SULFUR

The most important source of sulfur is SO_4^{2-} taken up by roots. Reduction of SO_4^{2-} is necessary for its incorporation into various biomolecules particularly cysteine (Leustek and Saito, 1999; Saito, 2000). Sulfur is a constituent of cysteine and methionine and thus acts as an important component of proteins. These Sulfur containing amino acids are the precursors for co-enzymes, intermediary metabolites and redox controllers. Sulfur is the structural constituent of several co-enzymes and prosthetic groups like ferredoxin, biotin and thiamine pyrophosphate. The SH groups act as functional groups in the enzyme reactions of urease, sulfotransferase and co-enzymes. Glutathione, a tripeptide (γ Glu-Cys-Gly) is the dominant nonprotein thiol in plants and is an important antioxidant in plants. Sulfur also promotes nitrogen fixation in legumes.

Genes encoding for sulfate transporters were isolated from barley (Vidmar et al., 1999) and Arabidopsis thaliana (Takahashi et al., 2000). Sulfur is required at a concentration of 0.1 to 0.5% of dry weight of plants for optimal growth. Sulfur deficiency decreased hydraulic conductivity of roots and net photosynthesis (Karmoker et al., 1991). Chlorophyll and protein content were also decreased in tomato under low levels of sulfur (Table 4). The growth of the shoot is more affected than root growth under sulfur deficiency. Thus shoot/root dry weight ratio decreased from 4.4 in sulfur sufficient

plants to 2.0 in sulfur deficient plants (Edelbauer, 1980). Sulfur deficiency induced accumulation of starch due to impaired carbohydrate metabolism (Willenbrink, 1967).

Table 4. *Influence of sulfur deficiency on chlorophyll, protein and starch content in tomato leaves**

Treatment	*Chlorophyll*	*Protein*	*Starch*
	mg/gm dry weight		
S-deficiency	0.9	3.5	27.0
Control (+SO_4^{2-})	5.8	48.0	2.8

**Adapted from Willenbrink (1967)*

Sulfur deficiency reduces the plant growth. The stems become thin and plants become rigid and brittle. The symptoms first appear on the top, leaves turn light yellow often followed by pronounced yellowing and all the leaves on the plant become light yellow to yellow. The sulfur deficiency symptoms may occur either in young leaves in the presence of sufficient nitrogen or in old leaves in the presence of low nitrogen (Robson and Pitman, 1983). This is because the remobilization and retranslocation of sulfur from the old leaves depends on the rate of nitrogen deficiency induced leaf senescence.

Plant growth is adversely affected if the SO_4^{2-} concentration is more than 50mM as occurs in some saline soils. The reduced growth rate and dark green colour of leaves are the characteristic features of sulfur toxicity.

Sulfur application to crops is becoming more common. Cereals require 30-40 kgs/ha/year where as members of Brassicaceae require more sulphur mostly for the synthesis of mustard oils. Gypsum, Super Phosphate, ammonium sulfate and potassium sulfate are important s-fertilizers.

6. CALCIUM

Calcium is a divalent cation (Ca^{2+}) and its content in soil varies depending on the parent material from which it is formed. In general, soils have sufficient Ca^{2+} in the soil solution to meet the crop demands and liming is done only to improve soil structure and pH. The plants have been adopted to varying conditions of pH and Ca^{2+} contents of different soils. Based on the tolerance to these conditions, plants could be categorized as calcicoles - the flora growing on calcareous soils and calcifuges - those growing on acid soils poor in calcium.

Calcium has different binding forms and is compartmentalized with in the cell. Higher concentrations of calcium is found in the cell wall, exterior plasma membrane,

endoplasmic reticulum and vacuoles. In the cytosol, calcium concentration is very low and maintained in the range of 0.1-0.2 μM (Evans et al., 1991). Calcium is bound as calcium pectate in the middle lamella and is essential for strengthening of cell walls and tissues in plants. Calcium is also required for root elongation. Calcium stabilizes cell membranes by bridging phosphate and carboxyl groups of phospholipids (Cladwell and Haug, 1981) and proteins (Legge et al., 1982) in the membrane. When calcium supply is low, 50% of total calcium can be bound as pectate in tomato (Armstrong and Kirkby, 1979b). Calcium stimulates α-amylase activity in germinating seeds (Bush et al., 1986).

In the cytosol, calcium binding proteins known as calcium modulated proteins like calmodulin (Snedden and Fromm, 2001), calmodulin binding proteins (Reddy et al., 2002) and calcium dependent but calmodulin independent protein kinases are the targets of calcium signals (Roberts and Harmon, 1992; Harmon et al., 2001). Calmodulin is an ubiquitous protein present universally in all eukaryotic cells. It is a polypeptide with 148 amino acids and four binding sites for calcium and it is heat stable and insensitive to pH changes. Calmodulin activates a number of enzymes like NAD kinase, adenylate cyclase and membrane bound ATPase.

Calcium requirement is lower in monocots than in dicots. For example 2.5 μM supply of calcium in rye grass and 100 μM of calcium in tomato are required for maximum growth (Table 5).

Table 5. *Effect of calcium on relative growth rates and calcium content in shoots of Rye grass and tomato**

Calcium conc.	*Calcium content (mg/gm dry wt.)*		*Relative growth rate*	
(μM)	*Rye grass*	*Tomato*	*Rye grass*	*Tomato*
0.8	0.6	2.1	42	03
2.5	0.7	1.3	100	19
10	1.5	3.0	94	52
100	1.7	12.9	94	100

**Adapted from Lonergan and Snowball (1969)*

Calcium deficiency is very rare if the pH is maintained properly. The deficiency symptoms first appear in growing tips and young leaves because of low mobility of Ca^{2+}. The tips and young young leaves become chlorotic followed by necrosis of leaf margins. Calcium deficiency induces premature shedding of fruit and buds. Excess calcium in the growth medium interferes with Mg^{2+} absorption. High Ca^{2+} usually causes alkaline pH which in turn precipitates many of the micronutrients making them unavailable to plants.

Liming plays an important role not only in amelioration of agricultural land but also in reclamation of waste heaps. Liming materials like $CaCO_3$, CaO and $Ca(OH)_2$ provide Ca^{2+} and induce an increase in pH due to their alkaline reactions. The heavy metals which are in higher concentrations in the waste material are phytotoxic under low pH conditions and the increase in pH with liming cause fixation of these otherwise toxic heavy metals (Wallace et al., 1966).

7. MAGNESIUM

Magnesium (Mg^{2+}) content varies in different soils. For example, sandy soils contain 0.05% and clay soils have 0.5% Mg^{2+} respectively. Magnesium occurs in three different forms such as exchangeable, non-exchangeable and water soluble forms. Water soluble and exchangeable Mg^{2+} are important sources of Mg^{2+} for plants.

The functions of magnesium in plants are related to its ability to interact with nucleophilic ligands (phosphoryl groups) through ionic bonding. Magnesium is also involved in regulation of cellular pH and cation-anion balance. Magnesium plays a significant role as the central atom of chlorophyll (Walker and Weinstein, 1991). Magnesium is essential for the aggregation of ribosome subunits (Cammarano et al., 1972) and for the synthesis of ATP (Lin and Nobel, 1971).

A number of enzymes like RNA polymerase, PEP carboxylase and glutathione synthetase require magnesium (Wedding and Black, 1988). Under Mg2+ deficiency RUBP-carboxylase activity is shifted in favour of RUBP-oxygenase due to accumulation of photosynthates in leaves and causing the formation of superoxide radicals (Cakmak and Marschner, 1992). Magnesium deficient leaves contained low levels of chloroplast pigments and associated with the accumulation of starch that results in the increase of dry matter yield (Table 6).

Table 6. *Effect of magnesium on chloroplast pigments and dry weight in Rape leaves**

Mg^{2+} level	*Dry weight (%)*	*Chlorophyll (mg/gm fresh wt.)*	*Carotenoids (mg/gm fresh wt.)*
Adequate	13.6	2.33	0.21
Inadequate	17.7	1.33	0.11

**Adapted from Baszynski et al., (1980)*

As Mg^{2+} is mobile, the deficiency symptoms first appear in the older leaves and then moves to young leaves. Interveinal chlorosis occurs in older leaves. Older leaves may become reddish-purple and the tips and margins become necrotic. Magnesium toxicity is not common. However, high Mg^{2+} content in the leaves are caused due to

drought conditions. Small necrotic spots occur in older leaves. If the toxicity is more the young leaves may exhibit spotted appearance.

Although soils are not deficient in Mg^{2+}, but with the high supply of other fertilizers rich in K^+ and NH_4^+, restrict the Mg^{2+} uptake by plants. Therefore, magnesium application becomes necessary. Sulphate fertilizers are more effective in this respect than carbonate fertilizers. In sandy soils, Mg^{2+} is applied at 80-160 Kg Mg^{2+}/ha which increase yield of various arable crops.

8. IRON

Iron is present in all types of soils making up about 5% by weight of earth's crust. The soluble iron content is very low in soils. Ferric (Fe^{3+}) and ferrous (Fe^{2+}) are soluble forms.

Iron is a transitional element and can change its oxidative state easily and form octahedral complexes with various ligands. Iron has two oxidation states – Fe^{2+} and Fe^{3+}. Metabolically active form of iron is Fe^{2+}, which is incorporated in to biomolecular structures.

Iron is a component of heme proteins, cytochromes, cytochrome oxidase, catalase, peroxidase, leghemoglobin and nonheme proteins like ferredoxin and lipoxygenase. Iron is also essential for the biosynthesis of chlorophyll (Pushnik and Miller, 1989). It plays an important role in biological redox systems and enzyme activation. Iron deficiency has significant effect on chloroplasts and protein content. The number of ribosomes decrease under its deficiency. Starch and sugar contents are low under iron deficiency. Low chlorophyll content, inhibition of photosynthetic electron transport with reduced regeneration of RUBP may be responsible for low starch and sugar content and low CO2 fixation in Fe-deficient plants (Sharma and Sanwal, 1992).

Plants can be categorized as iron efficient species (strategy I – dicots and non graminaceous monocots) and iron inefficient species (strategy II – Graminaceous monocots). Strategy I is characterized by increased reduction of Fe (III) to Fe (II) at the root surface. In addition, there is increased extrusion of protons due to the increased activity of the plasma membrane bound H^+ - ATPase. This rhizospere acidification also associated with the increased transmembrane electrical potential for the increased driving force for Fe (II) uptake (Brancardo et al., 1995). Strategy II is found in Poaceae members (grasses) under iron deficiency. These plants acquire Fe by releasing non protein amino acids which are called phytosiderophores (PS). Phytosiderophores form stable complexes with Fe III. This strategy also includes another component in the form of highly specific transport system which effectively transfers the Fe III – PS complexes in to the cytoplasm.

Iron deficiency is a world wide phenomena in crop production on calcareous soils. Deficiency symptoms first appear on young leaves which show interveinal chlorosis. In some cases, young leaves may be totally devoid of chlorophyll and are white in colour. In cereals, the leaves show yellow and green stripes along the length of the leaf.

Iron toxicity is mainly found in water logged soils and under flooded conditions. Iron toxicity may cause bronzing, stunted top and root growth.

Mulberry (*Morus alba* L) plants have shown increased dry matter yield upto 50.0 µM and a yield reduction was observed at 100.0 µM when grown in solution culture (Table 7).

Table 7. *Effect of ir on on dry matter yield and iron content in Morus alba L.var.kanva-2**

Iron supply leaves (µM)	*Dry matter yield (g plant^{-1})*	*Iron content in young (µg g^{-1} dry weight)*
0	36.84±1.45	110.39±7.50
5.0	36.47±4.55	120.13±5.62
50.0	86.77±5.72	146.10±3.75
100.0	70.90±2.05	137.98±0.94

**Adapted from Tewari (2004).*

Iron content in plant tissues is about 100 ppm of dry matter. Most of the agricultural crops require < 0.5 ppm in the soil in the plough layer where as total iron is about 2% or 20,000 ppm in the soil. The formation of iron-organic complexes, the chelates play vital role in supply of iron from soil to plants. Siderophores are recognized as the most important organic molecules which combine with iron and make it available to plants and microbes (Brown et al,. 1991).

9. MANGANESE

The soil content of manganese (Mn^{2+}) varies between 200 and 300 ppm. Availability of Mn^{2+} to plants depend on soil pH, microbial activity and soil water. The uptake of Mn is mainly suppressed by high levels of Fe, Zn and Cu.

Manganese is mainly associated with photosynthesis, redox processes and hydrolytic reactions. It forms strong complexes with biological ligands and participates in substrate-enzyme interactions of intermediary metabolism. In many biochemical functions Mn^{2+} resembles Mg^{2+}. Manganese can replace Mg^{2+} in many of the phosphorylating and group transfer reactions. Although there are several enzymes which are activated my Mn but mainly two Mn containing enzymes have been studied in detail. The first one is a manganoprotein – photosystem II of photosynthesis and the other one is superoxide dismutase (Elstner, 1982). Manganese activates several enzymes involved in the biosynthesis of some aromatic amino acids and certain secondary metabolites like lignin and flavonoids (Burnell, 1988).

Manganese-deficient plants contained low levels of soluble carbohydrates. The decrease is more in roots and this may be responsible for the reduced growth of roots (Marcar and Graham, 1987). Mn^{2+} deficiency induced the accumulation of soluble nitrogen whereas the protein content was more or less same in Mn^{2+} deficient and sufficient plants (Table 8).

Table 8. *Effect of manganese on growth and certain chemical constituents of bean plants**

Parameter	*Leaves*		*Roots*	
	+Mn	*-Mn*	*+Mn*	*-Mn*
Dry weight (g/plant)	0.64	0.46	0.21	0.14
Soluble carbohydrates (mg/g. dry weight)	17.50	4.00	7.60	0.90
Soluble Nitrogen (mg/g. dry weight)	6.80	11.90	17.20	21.70
Protein Nitrogen (mg/g. dry weight)	52.70	51.20	27.00	25.60

**Adapted from Vielemeyer et al. (1969).*

Manganese deficiency is common in soils with high pH and is also found in highly leached tropical soils. Mn deficiency symptoms first appear in younger leaves. The symptoms will vary from species to species. In monocots, greenish grey spots are seen in the basal leaves, resulting in chlorotic striping between the veins. The deficiency symptoms in oat is commonly referred as "grey speck" of oats. In dicots, initially small yellow spots are visible and later on develops in to interveinal chlorosis. The deficiency symptoms of Mn in certain crops were given specific names such as 'marsh spot of peas', 'grey speck of oats' and pahala blight of sugar cane'. Ohiki et al., (1981) reported critical deficiency level for most plant species in the range of 10-20 μg/g of dry matter.

Unlike the narrow range of manganese deficiency, the toxicity content varies between plant species and environmental conditions. The critical toxicity content range vary from 200 ppm in maize to 5300 ppm in sunflower (Table 9).

Table 9 . *Toxic levels of manganese in the shoots of cer tain crop plants**

Plant	*Manganese content (ppm)*
Maize	200
Pigeonpea	300
Soyabean	600
Cotton	750
Sweet potato	1380
Sunflower	5300

**Adapted from Edwards and Asher (1982).*

Normally manganese toxicity shows chlorosis in young leaves. This may be due to Mn^{2+} induced iron deficiency since Fe^{2+} deficiency also shows chlorosis in young leaves. In many plants the toxicity symptoms occur as interveinal chlorosis and necrosis. These symptoms are associated with deformation of young leaves and commonly known as "crinkle leaf".

Although many soils contain adequate levels of manganese, the application of Mn to calcareous peat soils is recommended. In these soils application of Mn fertilizers like $MnSO_4$ is usually applied.

10. ZINC

Zinc is the last element in the first transition series with completely filled 'd' orbitals. The zinc content of most of the soils is in the range of about 10-30 ppm. Plants take up zinc mainly as a divalent cation (Zn^{2+}).

The biochemical functions of zinc are based on its strong tendencies to form tetrahedral complexes with N, O_2 and S ligands and plays structural and functional role in enzyme reactions. Zinc is an activator of several enzymes, play an important role in DNA and RNA metabolism, protein synthesis, auxin metabolism and membrane integrity.

Carbonic anhydrase, alcohol dehydrogenase, superoxide dismutase, alkaline phosphatase, phospholipase and carboxy peptidase are important enzymes where zinc is an integral part of enzyme structure. Zinc activates a number of enzymes like dehydrogenases, aldolases and isomerases. Zinc plays an important role in DNA replication, transcription and regulation of gene expression (Coleman, 1992, Vallee and Falchuk, 1993).

Zinc deficiency decreases protein synthesis and results in the accumulation of aminoacids and amides. Further, zinc is essential for the activity of RNA polymerase and for the structural integrity of ribosomes. The ribosomes become unstable under

zinc deficiency and causes the decrease of protein content (Obata and Umebayashi, 1988). Zinc-deficient plants contained low levels of RNA. This may be due to higher activity of ribonuclease (Sharma et al., 1982). Low protein content in zinc deficient plants can be attributed to reduced levels of RNA (Cakmak et al., 1989). A close correlation between the zinc supply and nitrogenous fractions and RNA content was observed (Table 10).

Zinc deficiency is common in plants growing in highly weathered acid soils and calcareous soils. Higher phosphate levels also induce zinc deficiency. The most important symptoms of zinc deficiency include rosetting – stunted growth due to shortening of internodes, little leaf – decrease in size of leaves and die-back – death of shoot apices generally under severe deficiency of zinc. Interveinal chlorosis and necrosis observed in older leaves of zinc-deficient plants.

Zinc toxicity induces stunted growth, interveinal chlorosis in young leaves, which later on become dry and papery. Sometimes the affected leaves show the rolling of leaf margins. Roots turn brownish and necrotic. The critical toxicity levels in leaves of crop plants are 100 mg to 300 mg/g dry weight (Ruano et al., 1988).

Zinc uptake by crops is < 0.5 kg/ha/year. The deficiency could be corrected by spraying zinc salts like $ZnSO_4$ or by soil application of zinc chelates.

Table 10. *Effect of zinc on soluble nitrogen, protein nitrogen and RNA content of two varieties (CV.) of Cicer arietinum L **

CV.	*Zinc conc. (ppm)*	*Soluble nitrogen mg/g FW of leaves*			*Protein nitrogen mg/g FW of leaves*			*Soluble nitrogen as % of total nitrogen*			*RNA mg/g FW of leaves*		
		S1	*S2*	*S3*	*S1*	*S2*	*S3*	*S1*	*S2*	*S3*	*S1*	*S2*	*S3*
L-550	0	2.19	2.29	2.90	3.73	3.62	2.80	32.0	35.1	46.2	6.35	6.24	4.46
	0.011	1.96	1.85	2.44	4.74	5.21	4.45	25.7	23.0	31.5	7.64	8.78	6.82
	0.11	1.80	1.65	2.28	5.34	5.90	5.14	22.1	19.1	27.6	8.28	9.34	7.30
	0.22	1.78	1.57	2.31	5.50	6.02	5.16	21.6	18.2	27.7	8.26	9.43	7.24
	1.1	2.01	2.31	2.73	4.62	4.73	3.72	27.2	28.7	33.7	7.24	7.40	5.25
	0	2.32	2.38	2.92	3.56	3.36	1.73	35.6	37.5	56.6	6.12	5.85	3.72
	0.011	1.80	1.85	2.37	5.14	5.32	3.96	23.3	23.0	33.5	8.20	8.52	6.43
G-130	0.11	1.73	1.76	2.24	5.67	5.81	4.43	21.0	20.8	29.9	8.65	9.01	6.82
	0.22	1.70	1.82	2.29	5.72	5.83	4.28	20.4	21.2	31.1	8.76	8.98	6.75
	1.1	2.07	2.17	2.73	4.30	3.86	1.76	29.4	32.6	53.5	7.04	6.14	3.81

**Adapted from Reddy and Rao (1979).*
Mean square values on the basis of analysis of variance significant at 5% level
S1=Pre-flowering stage, S2=Flowering stage, S3=Post flowering stage

11. COPPER

Copper is a transition element and forms stable complexes. It occurs in two oxidation states (Cu^{+} and Cu^{2+}). Divalent form of copper is readily reduced to monovalent copper which is unstable. Copper occurs in soils mainly as a divalent cation. Copper is not easily mobile in plants but it is known to move from older to younger leaves and this movement depends on copper status of the plant.

Copper is an important redox component directly related to electron transfer reactions in photosynthesis, respiration, lignification of cell walls and detoxification of superoxide radicals (Fox and Guerinot, 1998). Copper is associated with three different forms of proteins (Sandmann and Boger, 1983) which include Blue proteins without oxidase activity (eg. Plastocyanin), Non-blue proteins which oxidize monophenols and Multi-copper proteins, containing around four copper atoms per molecule. (eg. Ascorbic acid oxidase and diphenol oxidase). Polyphenol oxidases are involved in the synthesis of lignin and alkaloids. Copper-deficient leaves of subterranean clover showed lower levels of polyphenol oxidase activity (Delhaize et al., 1985).

Nearly 50% of the copper localized in chloroplasts is associated with plastocyanin. Chlorophyll and plastocyanin levels and certain enzyme activities were more with the increased copper content in dry matter of *Pisum sativum* (Table 11)

Table 11. *Relationship between copper content, some constituents of chloroplast and activities of certain of certain enzymes in Pisum sativum leaves**

Copper (mg/g dry wt)	*Chlorophyll (m moles/g dry wt)*	*Plastocyanin (n moles/m mole chlorophyll*	*Diamine oxidase activity (m mole/g protein/hr)*	*Ascorbate oxidase activity*	*Superoxide dismutase activity (enzyme units/mg protein)*
2.2	4.4	0.3	0.24	220	3.6
3.8	3.9	1.1	0.43	470	13.5
6.9	4.9	2.4	0.86	730	22.9

** Adapted from Ayala and Sandmann (1988).*

Davies *et al.* (1978) observed that copper deficiency decreased the activities of polyphenol oxidase, IAA oxidase and peroxidase. Flowering was also affected by copper deficiency (Table 12).

Table 12 . *Effect of copper deficiency on flowering and certain enzyme activities In Chrysanthemum morifolium**

Treatment	*Copper content (mg/g leaf dry wt)*	*No. of flowering shoots / plant*	*Enzyme activity (relative)*		
			Polyphenol oxidase	*IAA oxidase*	*Peroxidase*
Cu deficient	2.4	8	26	52	41
Cu sufficient	7.9	14	100	100	100

**Adapted from Davies et al. (1978).*

The critical deficiency range of copper is about 1-5mg /g dry matter depending on plant species and growth stage and nitrogen supply (Thiel and Finck, 1973). In cereals, the deficiency symptoms first appear in the leaf tips at tillering stage. The leaf tips become white and leaves will be narrow and twisted. Plants show stunted growth in severe deficient conditions. Die back disease in citrus is very well documented. Legumes and tomato show interveinal chlorosis and distortion of the lamina.

Many crop plants show toxicity if the leaf tissue contains above 20 - 30 mg. Young leaves show interveinal chlorosis, while old leaves develop reddish orange or pink colouration and rolling of the leaf margins due to the loss of turgor. Later on, these leaves become dried and withered. Under severe toxicity, roots turn reddish brown and necrotic.

Foliar application of copper in the form of $CuSO_4$ or chelates are recommended to correct the copper deficiency in crops. Selection of genotypes which are efficient in high uptake of copper with more translocation efficiency from roots to shoots is another solution.

12. MOLYBDENUM

Molyndenum (Mo) is the only period five transition element required by plants in the form of molybdate oxyanion (MoO_4^{2-}). The total Mo content in most soils varies from 0.60 to 3.50 ppm. The available content is about 0.2 ppm. Molybdenum is required by plants in small quantities as compared to other elements except nickel.

Molybdenum is a constituent of nitrate reductase, nitrogenase, xanthine oxidase and sulphate oxidase. It plays a structural and catalytic role in these enzymes. Molybdenum is closely related to nitrogen metabolism and the requirement of Molybdenum depends on nitrogen source. Molybdenum deficient plants had shown higher activity of ribonuclease and lower activity of alanine transferase. Molybdenum deficiency induces the accumulation of organic acids and amino acids. Molybdenum deficient mustard leaves contained low levels of DNA and RNA (Chatterjee et al., 1985).

Molybdenum deficient plants are sensitive to low temperature stress and water logging (Vunkova - Radeva et al., 1988). Molybdenum deficiency affects flowering and pollen producing capacity. Pollen grain size and germination of pollen was also decreased under molybdenum deficiency (Table 13).

Table 13. *Effect of molybdenum on pollen production and viability in maize plants**

Molybdenum treatment (mg/kg)	*Molybdenum content of pollen grains (μg/g dry wt)*	*No. of pollen grains per anther*	*Pollen diameter (μM)*	*Pollen germination (%)*
0.01	17	1300	68	27
0.10	61	1937	85	51
20	92	2437	94	86

**Adapted from Agarwala et al. (1979).*

Molybdenum deficiency differs from species to species and also on the source of nitrogen supply. The threshold deficiency levels of Mo vary between 0.1 and 1.0 μg/g leaf dry weight (Bergmann, 1988). The visual symptoms are stunted growth and chlorosis in young leaves. Dicots are relatively susceptible to Molybdenum deficiency as compared to monocots. When Mo deficiency is severe, there will be a drastic reduction in leaf size and the development of irregular leaf lamina in cauliflower and this is commonly known as whiptail symptom. In molybdenum deficient bean plants, older leaves exhibit necrosis of the chlorotic areas between the veins and leaf margins.

When the molybdenum content exceeds 50 μg/g dry weight, there is a possibility of occurrence of Mo toxicity in many plants. Molybdenum toxicity induces the malformation of the leaves and often develop a golden yellow discolouration of the shoots due to the formation of molybdocatechol complexes. In potato and tomato the Mo toxicity causes the development of reddish or golden yellow colour of the shoots. Sulphate fertilization may reduce the molybdenum toxicity.

Many soils contain adequate levels of Mo to meet the requirement of crops. Molybdenum deficiency is seen in acid sandy soils and in soils with high anion exchange capacity. Legumes need more Mo to meet the demand of root nodule bacteria. Among the vegetables, cabbage and cauliflower are known to have higher demand for Mo. Molybdenum deficiency can be corrected by the foliar application of 100g $NaMoO_4$/ hectare. Sometimes liming also prevents Mo deficiency. Foliar application of Mo is beneficial as compared to soil application as evidenced by increase in the yield, nitrogen uptake and Mo content in groundnut, grown in Mo-deficient soil (Table 14).

Table 14. *Effect of molybdenum on dry matter yield, nitrogen uptake and molybdenum content in groundnut**

Molybdenum Treatment (g/ha)	*Dry matter (Kg/ha)*	*Nitrogen uptake (kg/ha)*	*Molybdenum content (µg/g dry wt.)*		
			Seeds	*Nodules*	*Shoots*
0	2685	70	0.02	0.4	0.02
200 (Soil application)	3413	90	0.02	1.5	0.20
200 (Foliar application)	3737	101	0.05	3.7	0.53

**Adapted from Rebafka et al., 1993*

An appropriate concentration of Mo has to be used not only to boost the agricultural yields but also to avoid reaching toxic levels of Mo accumulation in the forage plants. Whenever Mo content is above 5mg/kg dry weight of forage, it will induce toxicity in animals commonly known as "molybdenosis".

13. BORON

Boron is best considered as intermediate in properties between metals and non metals. Although soils contain around 20 to 200 ppm of boron (B), much of it is not available to plants because of it is held firmly by soil organic matter. Boron is commonly referred as an inert anion and available as boric acid. At lower pH it occurs as undissociated boric acid ($B(OH_3)$) and at higher pH it present as tetrahedral borate anion [$B(OH)^-_4$].

A significant proportion of total boron is complexed in cis-diol configuration in the cell walls of higher plants (Thellier et al., 1979). Boron forms cross links with certain polyhydroxy polymers like galactomannan (Loomis and Durst, 1991). Boron plays a key role in sugar transport and carbohydrate metabolism, cell wall synthesis, nucleic acid metabolism, phenol metabolism, IAA metabolism and membrane stability. For a long time it was suggested that boron plays an important role in the transport of sugars by the formation of sugar-borate complex (Dugger, 1983). This hypothesis is no longer acceptable since sucrose, the important sugar which is transported in the phloem, forms very weak complex with boron. Apart from this boron is not involved in the phloem loading of sucrose during sugar transport. The role of boron in cell wall synthesis and membrane integrity is evident in pollen tube growth (Vaughan, 1977). After germination new cell wall material is deposited at the tip of the pollen tube. If growing pollen tubes are deprived of boron, there was abnormal swelling in the tip region within 2-3 minutes of removal. Boron also influence fertilization by increasing the pollen grain

number and their viability (Agarwala et al., 1981). Boron is required for membrane integrity and function. Tanada (1978) had shown that boron is essential for the formation and maintenance of membrane potentials.

Boron deficiency induces callose formation and may block the sieve plate pores and results in the impairment of sugar transport. Boron deficient plants contained low levels of DNA and the synthesis of DNA was also decreased. Inhibition of DNA synthesis can be attributed to either a primary (Krueger *et al.*, 1987) or secondary (Ali and Jarvis, 1988) effect of boron deficiency. RNA content was also decreased in boron-deficient tomato plants. Dave and Kannan (1980) had shown that boron deficiency enhanced RNase activity in plants. So the decrease in RNA content can be correlated to higher activity of RNase.

Boron deficiency induces accumulation of certain phenolic compounds like caffeic acid and p-coumaric acid. Under boron deficiency, the carbon skeleton is shifted to pentose phosphate cycle and enhances the phenol production. Boron deficiency induces the accumulation of auxins. Necrosis in the growing points of boron-deficient plants is mainly due to the accumulation of auxins. Synthesis of cytokinins was depressed in sunflower roots under boron-deficient conditions (Wagner and Michael, 1971).

Boron deficiency reduced the phosphate uptake in field beans and it was restored when supplied with 10μM of H_3BO_3 for one hour (Robertson and Loughman, 1974). Boron deficiency is mainly seen in soils with high pH and under drought conditions. Boron deficiency symptoms first appear at the apical growing points or in young leaves. Young leaves become wrinkled and show darkish-blue green colour. Interveinal chlorosis may occur in mature leaves. The common boron deficiency symptoms are “top sickness” in tobacco, “heart rot” in sugar beet, “stem crack” in celary and “hollow stem” in cauliflower.

Boron toxicity is usually occurs in arid and semi arid regions. The toxicity may arise when boron fertilizers are used excessively to get more yields. The toxicity symptoms usually appear in young leaves which develop chlorosis between the veins. The leaf margins and the chlorotic areas become reddish brown. Later on the leaves become dry and papery. Flowering is also affected by boron toxicity.

There exists a narrow range between boron deficiency and toxicity, so care should be taken in applying the boron fertilizers to crop plants for increased yields. In general the application of boron varies from 0.3 to 3.0 kg/hectare depending on the requirement and sensitivity of crops to boron toxicity. Awarwala et al., (1969) had shown the critical level of boron deficiency, sufficiency and toxicity for certain crops (Table 15).

Table 15. *Threshold values of boron for deficiency, sufficiency and toxicity in certain crops**

Crop	Age of plant (days)	Boron content in leaves (ppm)		
		Deficiency	Sufficiency	Toxicity
Wheat (Kalyansona)	68	11	29-60	140
Maize (Ganga-101)	46	10	20-60	200
Paddy (IR-8)	48	18	20-72	400
Barley(K-19)	68	13	14-126	476

Adapted from Agarwala et al. (1969).

14. CHLORINE

Chlorine mainly found in soils as monovalent ion as chloride (Cl^-). Plants usually contain chloride in the range of 2 to 20 mg/g dry matter although the chlorine requirement for optimal growth is in the range of 0.20 to 0.40 mg/g dry weight. Chloride is available to plants from several sources like soil reserves, rain, irrigation water, fertilizers and through air pollution. Therefore there is much concern about chloride toxicity rather than deficiency in plants.

Chloride is mainly involved in the photolysis of water by photosystem II. Several experiments were conducted to show the evolution of oxygen by taking chloroplast fragments from different plant species. Chloride may either act as a bridging ligand for stabilization of the oxidized state of manganese (Critchley, 1985) or as a structural moiety of the extrinsic protein (Coleman et al., 1987). Proton pumping ATPase located in the plasma membrane is stimulated by monovalent cations particularly K^+ whereas ATPase at the tonoplast is not influenced by K^+ or any other cation but specifically stimulated by Cl^- (Churchill and Sze, 1984).

Chlorine plays an important role in the stomatal movement. Stomatal opening and closure is mediated by fluxes of K^+ and accompanying anions like malate and chloride (Raschke et al., 1988). Chloride has important functions in osmoregulation and plant water relations (Flowers, 1988). Due to its biochemical inertness chloride is able to fulfill osmotic and cation neutralization roles which may have biophysical or biochemical consequences of importance (Clarkson and Hanson, 1980).

Chlorine deficiency induces reduction in leaf area resulting in lower dry matter production. The decrease in leaf area is mainly due to reduction in cell division (Terry, 1977). Wilting of leaves is a typical symptom of Cl^- deficiency. In tomato, leaves show chlorotic mottling, bronzing and tissue necrosis. Wilting and premature senescence of leaves, frond fracture and stem cracking are general symptoms of Cl^- deficiency in coconut plants. When these plants were treated with KCl growth disorders disappeared to a larger extent with the increase in Cl^- content of leaves (Table 16).

Table 16. *Relationship between chloride content in leaves and growth disorders in coconut plants**

KCl application (Kg/plant)	*Leaf content (% dry wt.)*		*Growth disorders (%)*	
	K	*Cl*	*Frond fracture*	*Stem cracking*
0	1.61	0.07	11.6	27.0
2.25	1.64	0.41	1.7	8.1
4.50	1.66	0.51	1.2	4.5

**Adapted from Von Uexkull (1985).*

Chloride toxicity is a worldwide phenomenon and is an important factor limiting plant growth. The general symptoms are burning of leaf tips or margins, bronzing, premature yellowing and abscission of leaves. In some cases growth is reduced without any visual symptoms.

15. NICKEL

Nickel is a recently discovered micronutrient that can form chelate compound and can replace other heavy metals from physiologically important centres of metabolism. Most of the soils contain small quantities of nickel (< 100 ppm). Nickel is closely related to iron and cobalt in chemical and physiological properties. Although nickel may exist in the oxidation state of Ni(I) and Ni(II) but the most preferred oxidation state in biological system is Ni(II) (Cammack *et al*., 1988).

The function of nickel in higher plants was first time shown by Dixon et al., (1975) in connection with the urease activity. Eskew et al., (1984) reported that leguminous plants require nickel irrespective of the form of nitrogen source. Later, the essentiality of nickel for non-legumes was established (Brown et al., 1987). By these findings nickel is regarded as an essential element. Nickel is a component of urease, microbial dehydrogenases, hydrogenases and methyl reductase. It plays a significant role in urea and ureide metabolism, iron absorption, nitrogen fixation and seed development.

Nickel-deficient cowpea plants accumulated large amount of urea in tip of the leaf blade. Ureide levels were not affected by nickel supply (Walker et al., 1985). Brown et al., (1990) found that in barley the critical deficiency level of Ni is about 0.1 μg/g dry weight and associated with the accumulation of nitrate and amino acids. Nickel deficient plants show interveinal chlorosis and necrosis in leaves. Normally the nickel content of many plants is in the range of 1-10 μg/g dry matter. In some plants like lupin, nickel is preferentially translocated to seeds (Table 17).

Table 17. *Content of Nickel and certain micronutrients in shoots and seeds of Lupin and Rye (μg/g dry wt.)**

Plant	*Part*	*Ni*	*Fe*	*Mn*	*Zn*	*Cu*	*Mo*
Lupin	Shoots	0.81	178	298	28	3.6	0.08
	Seeds	5.53	47	49	41	6.0	3.29
Rye	Shoots	0.62	78	16	07	1.6	0.17
	Seeds	0.28	26	27	25	4.4	0.33

**Adapted from Horak (1985a).*

Crop plants are invariably subjected to nickel toxicity, when they are treated with sewage sludge. Critical toxicity levels are in the range of 10 μg/g in sensitive and 50 μg/g dry matter in tolerant plants (Asher, 1991). Plants suffering with nickel toxicity show necrosis on the leaf tips and margins. Young leaves may become severely distorted and the terminal shoot buds may die.

16. ENVIRONMENTAL ASPECTS OF MINERAL NUTRITION

Organisms are subjected to various environmental stresses. They try to adopt to these conditions for their physiological needs. Plants mainly depend on root system to acquire most of the mineral elements for growth and development. Roots are associated with variety of soil conditions ranging from arid to moist environment, different degrees of microbial activity and kinds of nutrient availability from acidic to basic. Plants are growing in a variety of soils where the pH of the soil solution may vary from extreme acidic conditions to alkaline situations.

About 50% of the world's arable land is acidic (Kochian et al., 2004). Aluminium (Al) availability increases in acid soils (< pH 5.5). Aluminium is one of the most abundant elements and constitutes about 8% of the earth's crust. Aluminium toxicity is an important growth-limiting factor for many plants. Detailed investigations have been carried out on the Al tolerance and toxicity in certain plants (Woolhouse, 1983). The primary target of Al toxicity is root growth, which is inhibited more than shoot growth (Fox, 1979). Root tips and lateral roots become thickened and turn brown. Al toxicity symptoms resemble phosphorus deficiency symptoms like stunted growth with dark green leaves, and yellowing and death of leaf tips. Al may interfere with phosphorus uptake and metabolism. At higher levels, aluminium induces Ca^{2+} and Mg^{2+} deficiency. Liming of acid soils decrease the risk of Al induced Ca^{2+} and Mg^{2+} deficiency and inhibition of root growth. Large number of plants in acid soils are adapted to Al toxicity. Foy (1974, 1975) found that certain cultivars of wheat, barley and soybean resisted Al

induced Ca^{2+} deficiency. Wheeler and his associates (1992c) screened 34 plants species for Al tolerance. Some plant species achieve Al tolerance by Al-activated exudation of organic acids from roots (Yang et al., 2000; Ryan et al., 2001). Aluminium tolerance genes were isolated from *Arabidopsis* and wheat (Sivaguru et al., 2003; Sasaki et al., 2004).

Soil salinity is a worldwide problem that consists of salt marshes of the temperate regions, adjacent to salt lakes and mangrove swamps. Saline soils are mainly found in semiarid and arid regions. Salinity is characterized by enrichment of salts, particularly sodium chloride, sodium sulfate and sometimes magnesium chloride and sulfate at the soil surface. Salinity usually occurs due to evapotranspiration causing a rise in ground water consisting of salts. Plant growth is severely affected when plants are subjected to salinity more than any other stress in the natural environment. Salinity affects the plant growth and development by impairing certain physiological and biochemical processes like water nutrient imbalance (Flowers et al., 1986), Photosynthesis (Yeo et al., 1985), Protein synthesis (Helal and Mengel, 1979) and phytohormone levels (Kuiper et al., 1990).

17. HEAVY METAL TOXICITY AND TOLERANCE

Heavy metals are defined as metals with a density higher than 5g cm^{-3}. Heavy metal pollution is posing several problems to mankind and these elements are increasing in the environment due to industrialization, mining and urban activity. Metals such as Hg, Pb, Cd, Al, Co, Mn, Ni, Cu and Zn are the important metals and their phytotoxicity is well documented (Wong and Bradshaw, 1982; Baker, 1987; Breckle, 1991). Metals like Cu, Mn, Ni and Zn are known as essential elements but when their concentration exceeds beyond a particular level they inhibit plant growth rather than promoting the growth and development. Research on metal toxicity and tolerance in plants has focussed on the potential and realized effects of a variety of metals on physiological and biochemical functions (Ernst, 1976; Foy et al., 1978; Taylor, 1988; Cumming and Taylor, 1990; Clemens, 2001).

Metal chelation is the most important mechanism and metal chelating compounds were first discovered in equine renal cortex by Margoshas and Vallee in 1957. Phytochelatins and metallothioneins are cysteine rich, heavy metal binding proteins. Phytochelatins are enzymatically synthesized polypeptides whereas metallothioneins are gene encoded polypeptides (Cobbett and Goldsruough, 2002). Grill et al., (1985) isolated phytochelatins from several plants. Phytochelatins are synthesized from glutathione (GSH). When plants are exposed to heavy metals, the synthesis of phytochelatins and simultaneous depletion of GSH was observed (Grill et al., 1985; Rauser, 1990; Cobbett, 2000). An excellent account of molecular biology of heavy metal stress in plants is given by Gasic and Korban (Chapter 8).

18. MOLECULAR BIOLOGY OF MINERAL NUTRITION

Earlier work on the genetic basis of mineral uptake and efficiency was initiated by plant scientists to check better varieties for cultivation of soybean by U.S.D.A. In soybean (Glycine max) Bernard and Howell (1964) found that a pair of alleles at a single locus named *Np* and *nP* control the response to high level of phosphate in the soils. Genotype NpNp showed little sensitivity to high phosphate (P-tolerant) as compared to other genotype having npnp being very sensitive (intolerant) to phosphate. Application of heavy doses of phosphorus fertilizers is accompanied by P-induced zinc deficiency. In such cases the need to look for genotypes from vast germplasm available should be made. The criteria for selection of genotypes should be those which give good yields with reference to

1. Adaptability to low P
2. Ability to gather P from low P soils
3. Responses to P
4. Tolerance to reasonably to high P without inducing Zn deficiency

Brown (1987) emphasized molecular approach to the problem of physiology of genotypic differences in Zn, Mn, Cu uptake in rice and tomato. The greater Zn and Cu uptake rates in rice was due to greater affinity of carrier sites in the root apices. In tomato, rapid uptake of Zn and Cu was due to greater capacity to absorb these ions due to higher concentrations of carrier molecules in roots. Ni et al., (1996) discusssed the genotypic differences in phosphorus stress induced changes in rice seedlings.

Novel approaches for genetic improvement of plant cultivars through recombinant DNA technology will throw up new avenues to tailor plants for better yields and efficient mineral nutrition. Isolation and characterization of genes involved in the uptake of minerals and their interaction within the various plant tissues will undoubtedly throw light on the mechanism operating in the optimum utilization of minerals for developing healthy and productive plants. Lee et al., (2003) found that transgenic *Arabidopsis thaliana* plants shown resistance to heavy metal stress with reduced uptake of lead and cadmium in heavy metal contaminated soils. For iron transport LeNramp1 protein was identified by Bereczky et al., (2003) in roots of tomato. Hussain et al., (2004) demonstrated that HMA_4 (transporter protein) was responsible for the transport of zinc in leaves of *Arabidopsis thaliana*. ShMTP1 located in the intracellular membrane of roots and leaves of *Stylosanthes hamata* was involved in the transport of manganese (Delhaize et al., 2003). Wintz and Vulpe (2002) found three copper chaperones in *Arabidopsis thaliana*. They suggested that these chaperones (metal receptors proteins) are involved in the uptake of copper as well as transport from cells.

Infact, efforts are underway to characterize structural and regulatory genes, signal transduction pathway, operating during ion mobilization by roots, their transport

to other tissues and metabolism. By adapting of *Arabidopsis thaliana* as an experimental model for genetic analysis, exciting work is in progress. It should be possible to develop plants with enhanced efficiency of nutrient acquisition and use, through genetic manipulation.

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CHAPTER 8

HEAVY METAL STRESS

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1. INTRODUCTION

Heavy metals are defined as those having densities higher than 5 g cm^{-3}. Ions of heavy metals such as iron, copper, zinc, cobalt, or nickel are essential micronutrients that are critically involved in functional activities of large numbers of proteins involved in sustaining growth and development of living organisms. However, at excess concentrations, these metal ions can become detrimental to living organisms. Furthermore, these organisms can be also exposed to highly toxic ions of cadmium, lead, mercury, and other metals that are generally considered non-essential.

It is apparent that complex network of transport, chelation, and sequestration processes has evolved over time that functions in maintaining concentrations of essential metal ions in different cellular compartments within a narrow physiological range, thus minimizing the damage caused by entry of non-essential metal ions into the cytosol (Halloran and Cullota, 2000; Clemens, 2001).

At the cellular level, plants have a wide range of potential mechanisms that are likely involved in the detoxification, and thus tolerance to heavy metal stress. Important components of heavy metal homeostasis and detoxification systems are membrane-based heavy metal transporters (reviewed by Williams et al., 2000), intracellular metal chaperones for efficient distribution of scarce essential metals, chelation (reviewed by Cobbett and Goldsbrough, 2002), and sequestration processes. Loss of any one of these critical processes will lead to hypersensitivity to heavy metal ions.

Depending on their oxidation states, heavy metals can be highly reactive, resulting in toxicity in most organisms. The ability of plants to increase antioxidative protection to combat negative consequences of heavy metal stress appears to be lim-

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ited, as it has been shown that exposure to elevated concentrations of redox reactive metals results in decreased rather than increased activities of antioxidative enzymes. The toxic effects of heavy metals on plants can be characterized by the following: (a) production of reactive oxygen species by autoxidation and the Fenton reaction, which is typical for transition metals such as iron and copper, (b) blocking of essential functional groups in biomolecules, which has been mainly reported for non-redox-reactive heavy metals such as cadmium and mercury, and (c) displacement of essential metal ions from biomolecules, which occurs with different kind of heavy metals (Schûtzendûbel and Polle, 2002).

2. METAL ACQUISITION

Understanding the processes involved in the movement of essential and non-essential metal ions into plant cells at the molecular level has vastly advanced in recent years. Associations between elevated steady-state transcript levels of metal transporter genes of the Zn-regulated transporter (ZRT), Fe-regulated transporter (IRT)-like protein (ZIP), and cation diffusion facilitator protein (CDF) families and metal hyperaccumulation (Pence et al., 2000; Lombi et al., 2002) or metal tolerance (Van der Zaal et al., 1999; Assunção et al., 2001; Persans et al., 2001) have been observed.

All heavy metal pumps, heavy metal ATPases (HMA), from bacteria, plants, and humans share significant sequence similarities, and cluster together as the P_{1B} subfamily (Palmgren and Axelsen, 1998). Ion pumps belonging to the P-type ATPase superfamily share a common enzymatic mechanism in which ATP hydrolysis is used to transport ions across a membrane. The "P-type" designation is attributed to a phosphorylated intermediate characteristic of the enzyme's catalytic cycle (Pedersen and Carafoli, 1987). Comparing rice (*Oryza sativa*) and *Arabidopsis* genome sequences has revealed similar numbers of P-type ATPase genes in both plant species, 43 and 46, respectively, despite the fact that the size of the rice genome is three times larger than that of Arabidopsis (Baxter et al., 2003). Both rice and *Arabidopsis* have representatives in all five major subfamilies of P-type ATPases. These include heavy-metal ATPases (P_{1B}), Ca^{2+}-ATPases (endoplasmic reticulum-type Ca^{2+}-ATPase and autoinhibited Ca^{2+}-ATPase, $P_{2A}$and P_{2B}), H^+-ATPases (autoinhibited H^+-ATPase, P_{3A}), putative aminophospholipid ATPases (ALA, P_4), and a branch with an unknown specificity (P_5). Genome sequence analysis of *Arabidopsis* has also revealed eight members of a 1_B ATPase subfamily of which four members are related to known Cu (I) transporters and contain N-terminal metal-binding site (MBS) motifs similar to those identified in other organisms. The remaining four members are more closely related to known divalent cation transporters in prokaryotes, three of which form a closely related group and are believed to be Zn (II) transporters (Cobbett et al., 2003).

Members of the ZIP gene family of metal transporters are capable of transporting a variety of cations, including cadmium, iron, manganese and zinc. The ZIP nomer is derived from the first identified members that include "ZRT (zinc regulated trans-

porter) and IRT (iron-regulated transporter) – like Proteins" (Guerinot, 2000). Many of the ZIP family genes are induced upon iron and zinc starvation, and their expression is mainly restricted to both roots and nodules (Fig. 1).

Among the first isolated transporter genes is the *Arabidopsis IRT1* gene, a major transporter responsible for high affinity iron uptake from the soil (Eide et al., 1996). Its abundance is controlled at both levels of transcript and protein accumulation. Overexpression of *IRT1* does not confer dominant gain-of-function enhancement of metal uptake. Iron deficiency results in induction of *IRT1* transcript accumulation; whereas, iron sufficiency results in reduction of *IRT1* transcript levels. High levels of zinc and cadmium also contribute to reduction in *IRT1* transcript levels. These findings suggest that the expression of *IRT1* is controlled by two distinct mechanisms that provide effective means of regulating metal transport in response to changing environmental conditions (Connolly et al., 2002). The selectivity of IRT1 is altered by replacing some of the amino acids in its residue (Rogers et al., 2000) allowing in the identification of those residues important for substrate selection and transport of a protein belonging to the ZIP gene family.

Cloning of the *IRT1* gene has led to the discovery of related members of the ZIP family. Complementation studies using a *Saccharomyces cerevisae* mutant, *zrt1 zrt2*, requiring elevated zinc levels in medium for growth confer Zn^{2+} uptake activity of Arabidopsis ZIP1-3 transporters (Grotz et al., 1998). *ZIP1* and *ZIP3* are expressed in roots in response to zinc deficiency, thus suggesting roles in transport of zinc from the soil into the plant. Whereas, *ZIP4*, identified by genome sequencing, is induced in both shoots and roots of zinc-limited plants, thus it may be involved in the transport of zinc either intracellularly or between plant tissues.

Studies on the effects of varying plant zinc ion status on both *ZNT1* expression and high–affinity Zn^{2+} uptake in roots of two *Thlaspi* species, the hyperaccumulator *T. caerulescens* and its related nonaccumulator *T. arvense*, have indicated that Zn hyperaccumulation in *T. caerulescens* is attributed, in part, by alteration in the regulation of Zn transporters by the plant Zn status (Pence et al, 2000). This alteration results in increased Zn transporter expression and a concomitant enhanced Zn^{2+} uptake and transport in the hyperaccumulating *Thlaspi* species. It has been demonstrated that an important component of the Zn hyperaccumulation trait in *T. caerulescens* involves overexpression of a Zn transporter gene, *ZNT1*, in both root and shoot tissues (Lasat et al., 2000). Later, Lombi et al. (2002) have investigated the effects of Fe status on Cd and Zn uptake and their influx in two *T. caerulescens* ecotypes, Ganges and Prayon. The *T. caerulescens* Zn- and Fe- regulated transporter-like protein genes, *TcZNT1-G* and *TcIRT1-G*, have been cloned from the Ganges ecotype and their expression have been analyzed under Fe–sufficient and –deficient conditions. Both, short- and long-term studies have revealed that Cd uptake is significantly enhanced by Fe deficiency in the Ganges ecotype, while Zn uptake is not influenced by the Fe status in either ecotype. This has indicated that the stimulatory effect of Fe deficiency on Cd uptake in Ganges may be

related to an up-regulation in expression of genes encoding for Fe^{2+} uptake, and possibly of that *TcIRT1-G*.

Nicotianamine (NA) is a metal chelator ubiquitously present in higher plants. In graminaceous plants, NA is a biosynthetic precursor of phytosiderophores, and it is therefore a crucial component in iron acquisition. In addition to their roles in phytosiderophore secretion from roots, three rice nicotianamine synthase genes, *OsNAS1*, *OsNAS2* and *OsNAS3*, have been found to play important roles in long-distance transport of iron in rice plants (Inoue et al., 2003). In maize, iron (III)-phytosiderophores are taken up by roots via YS1 transporters (Curie et al., 2001), belonging to the OPT oligopeptide transporter family (Fig. 1). Schaaf et al. (2004) have reported that ZmYS1 encodes a proton-coupled broad-range metal-phytosiderophore transporter that additionally transports Fe- and Ni-nicotianamine. These biochemical properties indicate a novel role for YS1 transporters for heavy metal homeostasis in plants.

Comparative analysis of gene expression profiles in *Arabidopsis* (Wintz et al., 2003) revealed specific transcriptional regulation by metals of genes contrasting with known wide-substrate specificities of encoded transporters. It was reported that two ZIP genes, *ZIP2* and *ZIP4*, were involved in copper transport; while, *AtOPT3*, a member of the oligopeptide transporter gene family with significant similarities to the maize iron–phytosiderofore YS1, was regulated by metals. Moreover, heterologous expression of *AtOPT3* could rescue yeast mutants deficient in metal transport.

Moreau et al. (2002) reported on the identification, characterization, and localization of GmZIP1, the first soybean member of the ZIP family of metal transporters. GmZIP1 encoded a symbiosis-specific zinc transporter in soybean. An antibody raised against GmZIP1 specifically localized the protein to the peribacteroid membrane, an endosymbiotic membrane in nodules resulting from the interaction of the plant with its microsymbiont. It was found that GmZIP1 possessed eight putative transmembrane (TM) domains together with a histidine-rich extra-membrane loop.

The two zinc transporters identified in rice showed many similarities in function, but they differed in expression pattern, ionic selectivity, and optimum pH for activity (Ramesh et al., 2003). One gene was widely expressed under all conditions, while the other gene was mainly induced upon exposure of plants to zinc deficiency and also to higher levels in roots than in leaves.

Another family of iron transporters, with homology to *Nramp* genes (Natural resistance associated macrophage proteins, TC 2.A.55), has been identified in plants. Thomine et al. (2000) carried out functional studies and characterized three members of the *Nramp* gene family in *Arabidopsis*. Their findings suggested that *AtNramp* genes encoded multispecific metal transport systems in plants that could transport Fe, Mn, and Cd^{2+} (Fig. 1). Following gene expression analyses of *AtNramp* in Arabidopsis it was reported that *Nramp* genes play roles in constitutive metal transport.

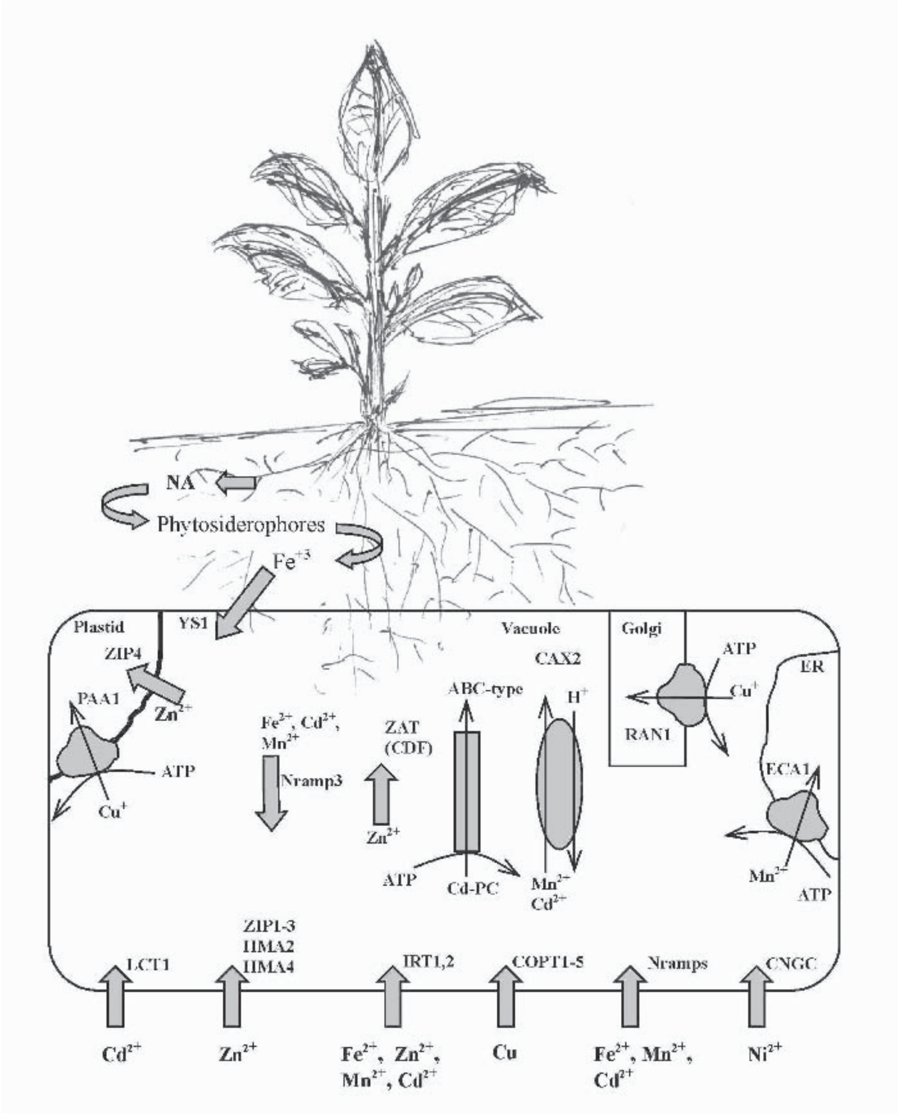

Figure 1. *Summary of putative metal transporters identified in plants today. Modified after Hall and Williams (2003)*

In *Pteris vittata*, the first identified arsenate (As) hyperaccumulator, arsenate is taken up via phosphate transporters, reduced to arsenite, and sequestered in fronds, primarily as As (III) (Wang et al., 2002). To investigate uptake, translocation, and speciation of As in roots and shoots of plants grown either in soil or in a nutrient solution, Quaghebeur and Rengel (2004) used two mutants of *A. thaliana*, *pho*, (P deficient) and *pho2* (P accumulator), defective in regulation and translocation of P (V) from roots to shoots. Shoots of the *pho2* mutant contained higher P concentrations, but either similar or slightly higher As concentrations than wild type. While for the *pho1* mutant, P levels were lower while As levels were higher in shoots than those found in wild type. Both *pho2* and wild type contained primarily As (III) in both roots and shoots (67-90% of total As). Arsenic was likely translocated to P (V) via different pathways in *pho2* and *pho1* mutants. Therefore, it has been suggested that As (III) was the main As species translocated from roots to shoots in *A. thaliana.*

The first plant protein that modulates plant tolerance or accumulation to Pb^{2+} was isolated and characterized (Arazi et al., 1999). Transgenic tobacco lines overexpressing a tobacco calmodulin-binding protein, NtCBP4, showed improved tolerance to Ni^{2+} and hypersensitivity to Pb^{2+} associated with reduced Ni^{2+} accumulation and enhanced Pb^{2+}accumulation, respectively.

Therefore, it is apparent that plants have developed various systems for essential metal ion uptake, and based on available evidence, it is believed that there are several mechanisms for uptake for each essential metal.

3. CHELATION

Once metal ions enter the cell, they are bound by chelators and chaperones. Chelators contribute to metal detoxification by buffering cytosolic metal concentrations; while, chaperones specifically deliver metal ions to organelles and metal-requiring proteins. There are several known metal-chelators in plants. These include phytochelatins, metallothioneins, organic acids, and amino acids (Clemens, 2001). Among heavy metal-binding ligands in plant cells, phytochelatins (PCs) and metallothioneins (MTs) are the best characterized, and these have been recently reviewed by Cobbett and Goldsbrough (2002).

3.1. Phytochelatins

Plants respond to excess heavy metal ions by synthesizing sulf-hydryl (SH)-containing peptides having the following general structure [(γ-$_L$-glutamyl-$_L$-cysteinyl)$_n$-glycine], where n = 2 to 11 (Rauser, 1990). Based on their structure and metal binding properties, these peptides are classified as class III metallothioneins, but the trivial name phytochelatins (PCs) has been widely adopted (Grill et al., 1985; Kägi, 1993).

PCs are enzymatically-synthesized directly from glutathione (GSH) by the enzyme PC synthase (EC. 2.3.2.15) (Grill et al., 1989; Cobbett, 2000) (Fig. 2). In plants, GSH

is enzymatically synthesized in two steps. In the first step, γ-glutamylcysteine synthetise (GCS) catalyzes formation of γ-glutamyl-cysteine, and in the second, GSH synthetase (GS) catalyzes the production of GSH through the addition of a glycine residue (Cobbett et al., 1998). In *Arabidopsis*, these two reactions are encoded by *GSH1* (May and Leaver, 1995) and *GSH2* (Wang and Oliver, 1996), respectively. GSH-deficient cadmium-sensitive mutants of *Arabidopsis* plants have been developed; these have either diminished capacity in producing gluthatione, *cad2* (Howden et al., 1995a) or *RML1* (Vernoux et al., 2000), or have a mutation in the gene coding for phytochelatin synthase, *cad1* (Howden et al., 1995b), thus synthesizing fewer phytochelatins resulting in hypersensitivity to both Cd and Cu. The enzyme PC synthase is constitutively expressed, but its activity is dependent on the presence of a heavy metal. Plant PC synthase genes have been cloned from wheat (*TaPCS1*) and *A. thaliana* (*AtPCS1*) (Clemens et al., 1999; Vatamaniuk et al., 1999). Later, it has been shown that PC synthase genes in wheat (Clemens et al., 1999) and in *Arabidopsis* (Lee and Korban, 2002) are regulated at the transcriptional level. However, transcriptional regulation of *AtPCS1* in *Arabidopsis* is observed only during early developmental stages, and it disappears as plants grow older (Lee and Korban, 2002). Molecular characterization of phytochelatin synthase expression in transgenic Arabidopsis has revealed its presence in leaves, roots, cotyledons, and stems, but not in root-tips or root hairs throughout all stages of plant development (Lee et al., 2002). Earlier, PC synthase has been reported to be present in roots and stems, but not in leaves or fruits of tomato plants (Chen et al., 1997).

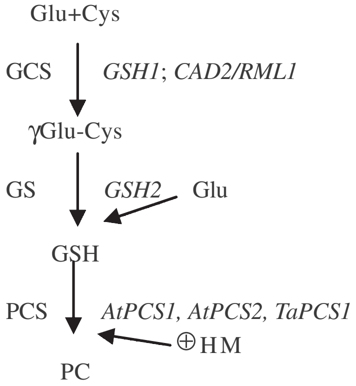

Figure 2. *Phytochelatin biosynthesis pathway*

Several attempts were made to alternate expression levels of enzymes involved in the phytochelatin biosynthesis pathway in plants (Strohm et al., 1995; Zhu et al., 1999a, b; Creissen et al., 1999; Arisi et al., 2000; Gisbert et al., 2003; Lee et al., 2003a, b) with varying success. Higher Cd tolerance and accumulation were observed when *E. coli* genes coding for either γ-glutamylcysteine synthetase (γ-ECS), *gshI*, or GSH syn-

thetase, *gshII*, were overexpressed in Indian mustard (Zhu et al., 1999a, b). Overexpression of the wheat PCS gene *TaPCS1* in *Nicotiana glauca* (shrub tobacco) also increased tolerance to metals such as Pb and Cd. Seedlings of transgenic plants grown in mining soils containing high levels of Pb accumulated twice the level of this heavy metal than wild type plants (Gisbert et al., 2003). Whereas, overexpression of *gshI* increased γ-ECS activity and accumulation of foliar glutathione in transformed poplar resulting in higher Cd accumulation in tissues, but had only a marginal effect on Cd tolerance in poplar (Arisi et al., 2000). On the other hand, transgenic tobacco plants overexpressing a chloroplast-targeted *gshI* exhibited a three-fold increase in foliar levels of GSH and enhanced oxidative stress (Creissen et al., 1999). Oxidative stress caused by gluthatione depletion, attributed to heavy metal induced phytochelatin synthesis, was reported in a number of plant species such as *Silene cucubalus* (De Vos et al., 1992) and *Holcus lanatus* L. (Hartley-Whitaker et al., 2001a). In a recent report, Lee et al. (2003a) observed that overexpression of AtPCS1 in Arabidopsis lead to hypersensitivity to Cd stress, which was attributed to the toxicity of phytochelatins at supra-optimal levels. Concurrently, transgenic Indian mustard plants overexpressing *AtPCS1* showed an increase in Cd tolerance and accumulation up to a certain level of heavy metal in the medium (100 μM $CdCl_2$), but as the level of Cd concentration increased (150-200 μM $CdCl_2$) severe inhibition in plant growth was observed (Gasic and Korban unpublished data). In contrast, when the *AtPCS1* was expressed in *Escherichia coli*, bacterial cells placed in the presence of heavy metals such as Cd or As showed 20- and 50-fold increase, respectively, in metal content (Sauge-Merle et al., 2003). Similarly, overexpression of a plant PC synthase gene in yeast resulted in enhanced resistance to arsenite as well as arsenate (Vatamaniuk et al., 1999).

In an attempt to understand the structural/functional organization of PC synthase, Rutolo et al. (2004) conducted a limited proteolysis analysis of the *Arabidopsis* AtPCS1 enzyme followed by functional characterization of the resulting polypeptide fragments. Their results suggested that AtPCS1 is composed of a protease resistant, presumably highly-structured, N-terminal domain, and flanked by an intrinsically unstable C-terminal region. The most upstream part of such a region, positions 284-372, appeared to be important for enzyme stabilization; while, its most terminal part, positions 373-485, was likely involved in determining enzyme responsiveness to a broad range of heavy metals.

The PC-based arsenic sequestration is essential for both normal and enhanced arsenate tolerance. Arsenate tolerance in *Holcus lanatus* requires both adaptive suppression of a high-affinity phosphate uptake system and constitutive phytochelatin production (Hartley-Whitaker et al., 2001a,b). Conversely, arsenate hypersensitivity in *Cytisus striatus* and grasses is based on reduced uptake through suppression of phosphate transporter activity (Bleeker et al., 2003).

Isolation of a PCS cDNA clone from *Brassica juncea* L. cv. Vitasso, a candidate plant species for phytoremediation, revealed a close relationship between BjPCS1 and PCS proteins from both *A. thaliana* and *T. caerulescens* (Heiss et al., 2003). When

BjPCS1 was expressed in *E. coli*, it exhibited a similar PCS activity *in vitro* in the presence of either 50 μM Cu or 200 μM Cd. Moreover, constitutive expression of *BjPCS1* during plant development was observed, and it was higher in roots, internodes, and petioles than in leaf tissues. Interestingly, prolonged Cd exposure resulted in a significant increase of PCS in leaves, but not in roots.

Using the γ-glutamylcysteine synthetase inhibitor L-butionine-[S,R]-sulphoximine (BSO), Schat et al. (2002) evaluated the role of phytochelatins in tolerance to Cu, Cd, Zn, As, Ni, and Co in non-metallicolous, metallicolous, and hypertolerant populations of *Sylene vulgaris* (Moench) Garcke, *T. caerulescens* J.&C. Presl., *Holcus lanatus* L., and *Agrostis castelana* Boiss. Et Reuter. It was observed that induction of PC accumulation decreased in the following order, As/Cd/Cu>Zn>Ni/Co. Moreover, it was consistently higher in non-metallicolous than in hypertolerant plants, except for As. PC-based sequestration was not essential for constitutive tolerance or hypertolerance to these metals nor for adaptive cadmium tolerance. Ebb et al. (2002) also reported that PCs did not play a role in the Zn/Cd hyperaccumulator *T. caerulescens* response to metal stress. Although PCs were produced by both the hyperaccumulator *T. caerulescens* and the non-accumulator *T. arvense*, PC levels in leaf and root tissues of both species showed positive correlation with Cd levels in these tissues, but they did not appear to be involved in metal tolerance in the hyperaccumulator *T. caerulescens*. In fact, total PC levels in *T. caerulescens* were generally low, despite their corresponding high metal concentrations, thus suggesting existence of other mechanisms responsible for Cd tolerance. While investigating tolerant plants collected from a copper mining dump, Leopold et al. (1999) showed that phytochelatins were not responsible for the development of metal tolerant phenotypes. Similar results were observed in transgenic Arabidopsis plants overexpressing *AtPCS1*, whereby higher levels of ectopic expression of Arabidopsis phytochelatin synthase did not lead to increased cadmium tolerance and accumulation (Lee et al., 2003b). In contrast, transgenic Arabidopsis plants with lower levels of *AtPCS1* expression demonstrated increased accumulation and tolerance to Cd compared to wild-type plant, and this was attributed to unknown toxic effects of PC (Lee et al., 2003b).

Gong et al. (2003) investigated long-distance root-to-shoot transport of phytochelatins and cadmium in Arabidopsis using wheat *TaPCS1* cDNA expressed either ectopically or in roots. Results showed that transgenic expression of TaPCS1 suppressed heavy metal sensitivity of the *cad1-3* mutant, and PC was transported from roots to shoots. Moreover, expression of *TaPCS1* increased long-distance root-to-shoot Cd^{2+} transport, and reduced Cd^{2+} accumulation in roots.

In addition to PCs with typical $(\gamma\text{Glu-Cys})_n$-Gly (*n*=2-11) amino acid sequences (Grill et al., 1986), several variant structures have also been detected. Homophytochelatins (iso-PC(βAla), $(\gamma\text{Glu-Cys})_n$-βAla, *n*=2-7) were first isolated from the Phaseoleae (Fabaceae) family (Grill et al., 1986; Klapheck et al., 1995; Oven et al., 2001), and isophytochelatins (Ser) (iso-PC(Ser), $(\gamma\text{Glu-Cys})_n$-Ser, *n*=2-4) have been detected in species of the Poaceae family (Klapheck et al., 1995). A homologue of glu-

tathione with a C-terminal linked Glu instead of Gly as well as isophytochelatins (Glu) (iso-PC(Glu), $(\gamma\text{Glu-Cys})_n$)-Glu, *n*=2-3) (Meuwly et al., 1993, 1995) and desGly-PC homologues lacking an N-terminal γ-linked Glu (Chassaigne et al., 2001) were isolated from maize plants exposed to cadmium. Additionally, desglycine phytochelatins (des Gly-PC, $(\gamma\text{Glu-Cys})_n$), lacking a C-terminal amino acid residue, were first discovered in maize (Bernhard and Kägi, 1987). PC-related peptides, including isophytochelatins (Gln) (iso-PC(Gln) (γGlu-Cys)*n*-Gln, *n*=3-4), were identified in horseradish hairy roots exposed to cadmium (Kubota et al., 2000). Oven et al. (2002b) reported on the isolation and functional characterization of a homo-phytochelatin synthase GmhPCS1 from *Glycine max*, a plant known to accumulate homo-phytochelatins rather than phytochelatins upon exposure to heavy metals. The catalytic properties of GmhPCS1 were compared with those of the phytochelatin synthase AtPCS1 from *A. thaliana*. The incorporation of both gluthatione and homoglutathione into homophytochelatin, n=2, was demonstrated using GmhPCS1 and AtPCS1. These findings suggested that the presence of the GSH substrate and its' conforms and not the specificity of the enzyme determines the nature of PCs synthesized in any given plant. In addition to bis(glutathionato)•metal complexes, various other metal•thiolates were shown to contribute to the activation of phytochelatin synthase. Previously, it has been reported that metal binding *per se* was not responsible for catalytic activation of AtPCS1. Moreover, the dependence of AtPCS1 on heavy metal ions for activity in media containing GSH and other thiol peptides was a reflection of this enzyme's requirement for glutathione-like peptides containing blocked thiol groups for activity (Vatamaniuk et al., 2000). However, findings of Oven et al. (2002b) suggested that the proposed model for AtPCS1 activation could not fully explain the catalytic mechanism of phytochelatin synthase.

Activation of the detoxicative-phytochelatin system was observed in the cytosol of root cells of three plant species, *Vicia faba*, *Pisum sativum,* and *Phaseolus vulgaris*, belonging to the Fabacea family and exposed to lead ions (Piechalak et al., 2002). This system was composed of phytochelatins (PCs) in roots of *V. faba*, homophytochelatins (hPCs) in *P. vulgaris* roots, and both PCs and hPCs in *P. sativum* roots.

Therefore, current results on PCs and their role in heavy metal stress in plants show that PCs are synthesized in plants in response to heavy metal stress and due to various metals. However, analysis of naturally occurring heavy metal hyperaccumulating plants has demonstrated involvement of some other mechanisms, yet be elucidated, that allow these plants to survive on contaminated soils and accumulate excess heavy metals.

3.2. Metallothioneins

Metallothioneins (MTs) belong to a family of gene-encoded low-molecular weight cysteine-rich metal-binding proteins (Robinson et al., 1993). Although, the precise physiological function of MTs have not yet been fully elucidated, proposed roles include the

following: (a) participation in maintaining the homeostasis of essential transition metals; (b) sequestration of toxic metals, such as cadmium and mercury; and (c) protection against intracellular oxidative damage. MTs are involved in heavy metal homeostasis and tolerance to Cd^{2+} and Zn^{2+} in mammals (Masters et al., 1994) and to Cu and Cd^{2+} in yeast (Hamer et al., 1985; Yu et al., 1994). The first plant MT involved in Zn^{2+} binding, E_c (early Cys-labelled) protein, was identified in wheat (Lane et al., 1987). Since then, more than 50 MT-like sequences have been found in plants (Rauser, 1999).

Isolation and characterization of type 1 metallothionein cDNA (*mcMT1*) from a heavy metal tolerant plant, *Festuca rubra* cv. Merlin, was recently reported (Ma et al., 2003). Functional complementation studies using the *Saccharomyces cerevisiae cup1*Δ mutant ABDE-1 (metal-sensitive) confirmed the functional nature of this *mcMT1* gene in sequestering both essential (Cu, Zn) and non-essential metals (Cd, Pb, Cr).

Expression and regulation of Arabidopsis MT genes have revealed that plant MTs have distinct functions in metal homeostasis, especially for Cu (Murphy et al., 1997; Garcia-Hernandez et al., 1998; Guo et al., 2003). *MT1a* and *MT2b* are involved in the distribution of Cu via the phloem; while, *MT2a* and *MT3* chaperone excess metals in mesophyll cells and root tips. These functional capabilities may allow MTs to play roles in mobilization of metal ions from senescing leaves and sequestration of excess metal ions in trichomes. An Mt2b-like gene was identified in copper tolerant *Silene vulgaris* (Moench) Garcke (van Hoof et al., 2001). When expressed in yeast, this gene, *Sv2b*, restored tolerance to cadmium and copper in different hypersensitive strains.

Two distinct class I type 3 metallothionein-like genes, *MT3-A* and *MT3-B*, were isolated from oil palm (Abdullah et al., 2002). Both *MT3* genes were expressed in the mesocarp throughout the ripening period. Moreover, *MT3-B* was also expressed in roots while *MT3-A* was induced in senescing leaves.

Isolation of a cDNA sequence encoding a type 2 metallothionein (MT)-like protein, htMT2, from *Helianthus tuberosus L.* was reported by Chang et al. (2004). This protein was mainly present in internodes and nodes, but was not detected in roots. It was suggested that HtMT2 might be involved in either transport or availability of Cu^{2+} and Zn^{2+} to some apometal enzymes or apometal proteins.

3.3. Organic Acids and Amino Acids

The presence of different concentrations of organic acids among various ecotypes of metal-tolerant plants in their natural habitat has deemed these substances as likely cellular chelators (Rauser, 1999). Studies have demonstrated that the primary constituents of root exudates are low-molecular weight organic acids that play essential roles in making sparingly soluble soil Fe, P, and other metals available to growing plants (Römheld and Awad, 2000; Yang and Crowley, 2000).

Various plant species have developed mechanisms to enable them to grow on acid soils where toxic levels of aluminum, Al^{3+}, can limit plant growth. Some plants

detoxify aluminum in the rhizosphere by releasing organic acids that chelate aluminum, while others detoxify aluminum internally by forming complexes with organic acids (Ma et al., 2001). Extracellular chelation by organic acids, such as citrate, oxalate, and malate, is important in aluminum tolerance. Malate is released from roots of Al-tolerant cultivars of wheat (*Triticum aestivum*); while, citrate is released from roots of Al-tolerant cultivars of snapbean (*Phaseolus vulgaris*), maize (*Zea mays*), *Cassia tora*, and soybean (*Glycine max*). Whereas, oxalate is released from roots of buckwheat (*Fagopyrum esculentum*) and taro (*Colocasia esculenta*) (Delhaize et al., 1993; Pellet et al., 1995; Ma et al., 1997a; Yang et al., 2001; Ma et al., 1997b; Ma and Miyasaka, 1998). Some plant species, such as Al-tolerant triticale (x *Triticosecale* Wittmack), rapeseed (*Brassica napus*), oat (*Avena sativa*), radish (*Raphanus sativus*), and rye (*Secale cereale*) release both malate and citrate (Ma et al., 2000; Zheng et al., 1998; Li et al., 2000).

Sasaki et al. (2004) have cloned a wheat gene, *ALMT1* (aluminum-activated malate transporter), that co-segregates with Al tolerance in F_2 and F_3 populations derived from crosses between near-isogenic wheat lines that differ in Al tolerance. The *ALMT1* gene codes for a membrane protein constitutively expressed in root apices of an Al-tolerant line at higher levels than in a near-isogenic Al-sensitive line. Heterologous expression of *ALMT1* in *Xenopus* oocytes, rice, and cultured tobacco cells has conferred an Al-activated malate efflux. Additionally, *ALMT1* has increased tolerance of tobacco cells to Al treatment. These findings demonstrate that *ALMT1* codes for an Al-activated malate transporter capable of conferring Al tolerance to plant cells.

It has been reported that Al treatment in Arabidopsis induces oxidative stress genes (Richards et al., 1998). Ezaki et al. (2001) have characterized the mechanism of action of four genes, including *AtBCB* (Arabidopsis blue copper-binding protein), *parB* (*Nicotiana tabacum* gluthatione *S*-transferase), *NtPox* (tobacco peroxidase), and *NtGDI1* (tobacco GDP dissociation inhibitor), involved independently in Al resistance using transgenic Arabidopsis lines. Apparently, these four genes have different biochemical functions, thus suggesting that there are several different mechanisms for Al tolerance in plants in addition to the release of organic acids. Influx and efflux experiments of Al ions have suggested that the *AtCBC* gene likely suppresses Al absorption; whereas, expression of the *NtGDI1* gene promotes the release of Al in root-tips of Arabidopsis plants. Overexpression of *parB* and *NtPox* diminishes oxidative damage caused by Al stress. Analysis of F_1 hybrids among the four transgenic lines suggests that enhanced resistance can be achieved by combining some of these four genes in individual lines.

Exposure of plant cells of the cobalt hyperaccumulator *Crotalaria cobalticola* and non-accumulators *Raufolia serpentine* and *Silene cucubalus* to cobalt ions have resulted in increases of both citrate and cysteine suggesting that these two proteins are involved in cobalt ion complexation (Oven et al., 2002a).

Under heavy metal stress, a high cysteine biosynthesis rate is required for the synthesis of GSH and phytochelatins. *O*-acetyl-serine(thiol)lyase (OASTL) is a key enzyme of a plant sulfur metabolism that catalyses the formation of Cys which serves as

a precursor for GSH. Transgenic tobacco plants expressing a wheat OASTL gene, *cys1*, show increased cysteine biosynthesis and involvement in plant responses to oxidative stress (Youssefian et al., 2001). Domigues-Solis et al. (2001) have reported that an Arabidopsis *OASTL* gene, *Atcys-3A*, is involved in cadmium tolerance. Transgenic tobacco plants overexpressing cysteine synthase in both the cytosol and the chloroplast showed significant increases in tolerance for Cd, Se, and Ni supplemented in an agar medium (Kawashima et al., 2004). F_1 transgenic plants demonstrating higher resistance towards these metals also showed enhanced accumulation of Cd in shoots.

Several studies proposed the involvement of either organic or amino acid chelation in enhancing the rate of root-to-shoot transport of transition metal ions (Lee et al., 1977; White et al., 1981; Senden and Wolterbeek, 1992; Krämer et al., 1996; Liao et al., 2000). Correlations were observed in xylem sap concentrations between copper and nicotianamine (Pich et al., 1994; Liao et al., 2000), and between copper and histidine (Liao et al., 2000). As a result, it has been suggested that both amino acids, nicotianamine and histidine, were involved in chelation of copper ions in the xylem sap.

Some amino acids, particularly histidine and proline, also play roles in the chelation of metal ions both within plant cells and in the xylem sap (Rai, 2002). Exposure of the hyperaccumulator *Alyssum lesbiacum* to nickel (Ni) is known to result in a dose-dependant increase in xylem sap concentrations of both Ni and the chelator-free histidine (His). Recently, Kerkeb and Krämer (2003) have reported that an enhanced release of Ni into the xylem is associated with concurrent release of His from an increased root-free His pool. Particularly in *B. juncea* roots, Ni^{2+} uptake is independent of simultaneous uptake of His.

Many plants have been reported to accumulate proline (Pro) when exposed to heavy metals (Alia and Saradhi, 1991; Basi and Sharma 1993a,b; Costa and Morel 1994; Talanova et al., 2000). Siripornadulsil et al. (2002) have demonstrated that increased Pro levels provide enhanced protection against Cd in microalgae. It is interesting to note that Pro reduces Cd stress not by sequestering Cd, but by reducing Cd-induced free radical damage and maintaining a stringent reducing environment (higher GSH levels) within the cell.

The chelation strategy involves extrusion of low-molecular mass secondary amino acids (mugineic acids), known as "phytosiderophores", that chelate the sparingly soluble iron. In plants, metal phytosiderophore uptake is known to be important for supplying the demands for micronutrients. Among higher plants, graminaceous species are reported to secrete phytosiderophores, hexadentate metal chelators with high affinity for Fe (III), to efficiently acquire iron (Fe) from alkaline soils with low Fe solubility (Schaaf et al., 2004). The phytosiderophore system in plants serves the acquisition of Fe and other metals as well. It has been shown that phytosiderophore release is also triggered under conditions of Zn deficiency (Tolay et al., 2001). Although, with high affinity for ferric iron, phytosiderophores of the mugineic acid family also chelate other heavy metals, such as Cu, Mn, and Zn as well as other non-essential metals (Treeby et al., 1989; Römheld and Awad, 2000).

Iron chelate reductases or the ferric reductase oxidase (FRO) protein family are known to be responsible for the ability of plants to change the redox state of Fe^{+3} in the soil as a first step in iron uptake (Fig. 2). These have been detected in *Arabidopsis* (Robinson et al., 1997; Robinson et al., 1999), pea (*Pisum sativum*) (Waters et al., 2002), and rice (Gross et al., 2003).

Nicotianamine (NA), a non-proteinaceous amino acid, is ubiquitously present in higher plants, and it is known to be involved in chelation of metals. Nicotianamine aminotransferase (NAAT) catalyzes the amino group transfer of NA in the biosynthetic pathway of phytosiderophores, and it is essential for iron acquisition in graminaceous plants. In addition to its role in long-distance metal transport, NA is proposed to be involved in the regulation of metal transfer within plant cells (Pich et al., 2001; Takahashi et al., 2003). On the other hand, nicotianamine synthase (NAS) is an enzyme that is critical for the biosynthesis of the mugineic acid family of phytosiderophores in graminaceous plants and for homeostasis of metal ions in nongraminaceous plants. Mizuno et al. (2003) have revealed that maize has two types of NAS proteins, based on their expression pattern and subcellular localization. A total of three NAS genes have been isolated from maize, one genomic clone, *ZmNAS3*, and two cDNA clones, *ZmNAS1* and *ZmNAS2*. Both *ZmNAS1* and *ZmNAS2* are expressed only in Fe-deficient roots. This is in agreement with the reported increased secretion of phytosiderophores. In contrast, *ZmNAS3* is expressed under Fe-sufficient conditions, and it is negatively regulated by Fe-deficiency, thus suggesting a role different than that of a precursor in phytosiderophore production. Although ZmNAS1-green fluorescent protein (sGFP) and ZmNAS2-sGFP have been localized in the cytoplasm of onion epidermal cells, ZmNAS3-sGFP is distributed throughout the cytoplasm of these cells.

The hyperaccumulation of zinc (Zn) and cadmium (Cd) is a constitutive property of the metallophyte *A. halerii*. Recently, Weber et al. (2004) have used Arabidopsis GeneChips to identify those genes that are more active in roots of *A. halerii* than *A. thaliana* under controlled conditions. Two genes showing highest levels of expression in *A. halerii* roots code for a nicotianamine (NA) synthase and a putative Zn^{2+} uptake system. In addition, roots of *A. halerii* also show higher levels of both NA synthase and NA. Expression of NA synthase in Zn^{2+}-hypersensitive *Schizosaccharomyces pombe* cells has demonstrated that formation of NA can confer Zn^{2+} tolerance. Taken together, these observations suggest active roles of NA in plant Zn homeostasis and NA synthase in hyperaccumulation of Zn in *A. halerii*.

On the other hand, algae respond to heavy metals by induction of several antioxidants, including diverse enzymes such as superoxide dismutase, catalase, glutathione peroxidase, and ascorbate peroxidase, as well as synthesis of low-molecular weight compounds such as carotenoids and glutathione (Pinto et al., 2003). Boominathan and Doran (2003) demonstrated that metal-induced oxidative stress occurred in tissues of the hyperaccumulator *T. caerulescens* even though growth was unaffected in the presence of heavy metals. They also suggested that superior antioxidative defenses, particularly catalase activity, might play important roles in the hyperaccumulator phe-

notype. Increased activities of antioxidative enzymes in Cd-treated plants and their involvement in mechanisms of metal tolerance were also reported in pea (*Pisum sativum* L. cv. Azad) (Dixit et al., 2001).

4. METAL TRAFFICKING

Transition metals such as Fe, Cu, Mn, and Zn are essential minerals for normal plant growth and development, although they can be toxic when present at excess levels. Thus, for healthy plant growth, a range of transition metals must be acquired from the soil and distributed within the plant, but their concentrations must be carefully regulated within different cells and organelles. Membrane transport systems are likely to play a central role in these processes. The application of powerful genetic and molecular techniques has now identified a range of gene families that are likely to be involved in transition metal transport. These include heavy metal ATPases (HMAs), Natural resistance associated macrophage proteins (Nramps), the cation diffusion facilitator (CDF) family, the ZIP family, and cation antiporters (Table 1). An overview of the broad range of potential transport systems likely involved in uptake, distribution, and homeostasis of transition metals in plants has been published by Hall and Williams (2003).

4.1. Intracellular Sequestration

As excess metal ions accumulate within the plant cell, they have to be removed from the cytosol. This is usually done by either efflux or compartmentalization. The main storage organelle for toxic compounds in plant cells is the vacuole. The central vacuole is the largest compartment of a mature cell, and may occupy more than 80% of the total cell volume. In addition to the large central vacuole, several small vacuoles may exist within a plant cell. In fact, two vacuolar proton pumps, an ATPase and Ppas, energize vacuolar uptake of most solutes. Uptake can be catalyzed by either channels or transporters. For most ions, more than one transporter/channel exists at the vacuolar membrane (Martinoia et al., 2000) (Fig. 1).

Vacuolar compartmentalization or cell wall binding in leaves could play major roles in hyperaccumulation of heavy metals. Cosio et al. (2004) investigated the role of leaf cells in allocating metals in such hyperaccumulating plants as *T. caerulescens* “Ganges” and *A. halerii*, both hyperaccumulators of Zn and Cd, and to a lower extent in *T. caerulescens* “Prayon”, an accumulator of Cd. Their work demonstrated that cellular uptake of Cd and Zn in leaf cells of hyperaccumulating plants involved a carrier-mediated mechanism. Moreover, differences in metal uptake among *T. caerulescens* “Ganges”, *T. caerulescens* “Prayon”, and *A.* halleri could not be explained by differences in transport capacities of tonoplasts in leaves. Therefore, they concluded that regulation mechanisms must be present before the plasma membrane of a leaf cell that directed metals to their final location within a cell. Moreover, as the accumulation capacity of protoplasts was modified after a plant’s pre-exposure to Cd, this indicated

that once plants were exposed to a certain threshold level of Cd, different transport mechanisms were induced in leaf cells. Carrier et al. (2003) investigated Cd distribution and microlocalization in oilseed rape (*Brassica napus*) following long-term growth on Cd-contaminated soil. Microanalytical results confirmed that long-term growth of *B. napus* on Cd-contaminated soil was accompanied by preferential storage of Cd in both vacuoles and cell walls. This phenomenon diverted Cd ions from metabolically active compartments (cytosol, chloroplast, mitochondria), thus resulting in reduction of Cd toxicity in leaves.

Küpper et al. (2004) reported on tissue- and age-dependent differences in the complexation of Cd and Zn in the hyperaccumulator *T. caerulescens*. They indicated that the type of ligand complexing with Cd and Zn depended on the metal as well as both the function and age of the plant tissue. For example, in mature and senescent leaves, oxygen ligands were dominant. This finding, combined with our earlier knowledge of metal compartmentalization, indicated that plants preferred detoxifying hyperaccumulated metals by pumping them into vacuoles rather than synthesizing metal-specific ligands. In young and mature tissues (leaves, petioles, and stems), a higher percentage of Cd was bound by sulfur (S) ligands (e.g., phytochelatins) than in senescent tissues. This likely indicated that young tissues required strong ligands for metal detoxification in addition to sequestration of metals in epidermal vacuoles. Alternatively, this might shed some light on the known smaller proportion of epidermal metal sequestration going on in younger tissues in combination with presence of steady high proportions of S ligands in mesophylls. In stems, higher proportions of Cd and Zn were coordinated by S ligands and histidine, respectively, compared with leaves of the same age. This suggested that metals were either transported as stable complexes or that vacuolar oxygen coordination of metals was mainly taking place in the epidermis as observed in leaves. The epidermis constitutes a larger percentage of the total volume in leaves than in either stems or petioles. As Zn-S interaction was not observed, this confirmed earlier results reporting that S ligands were not involved in Zn resistance in hyperaccumulator plants.

Analysis of cellular compartmentalization of elements in the Zn hyperaccumulator *A. halerii* (L.) “O’Kane” and “Al-Shehbaz” revealed not only Zn, but also Cd hyperaccumulation in shoot biomass (Küpper et al., 2000). High levels of Zn and Cd were found in leaves (trichomes) and roots (rhizodermis), but very little in flowers. Mesophyll cells in leaves of *A. halerii* appeared to be major storage sites for Zn and Cd, and played important roles in metal hyperaccumulation. In addition, when Ni uptake and compartmentalization were investigated in three nickel hyperaccumulators, *Alyssum bertolonii* (Desv), *A. lesbiacum* (Candargy), and *T. goesingense* (Hálácsy), Ni was preferentially distributed in epidermal cells, and most likely in vacuoles of leaves and stems (Küpper et al., 2001).

Table 1. *Proposed specificity and location of the major metal transporters in plants*

Name	Source	Proposed specificity	Cellular location	Main tissue expression	References
Chaperones					
CCH	*A. thaliana*	Cu		Phloem	Himelblau and Amasino 2000; Mira et al., 2001.
Heavy metal ATPases (P_{1B})					
RANI (AtHMA7)	*A. thaliana*	Cu	Post-Golgi	Whole plant	Hirayama et al., 1999; Woeste and Kieber 2000.
PAAI (AtHMA6)	*A. thaliana*	Cu	Chloroplast		Shikanai et al., 2003.
HMA2	*A. thaliana*	Zn	Plasma membrane	Roots, stems and leaves	Hussain et al., 2004.
HMA4	*A. thaliana*	Zn		Roots stems and leaves	Hussain et al., 2004.
Ca^{+2}-ATPases (P_{2A})					
ECA1	*A. thaliana*	Mn^{2+}	ER	Roots?	Wu et al., 2002.
Nramp					
AtNramp 1	*A. thaliana*	Fe, Mn		Roots	Willaims et al., 2000;
AtNramp 2	*A. thaliana*	-		Roots	Maser et al., 2001.
AtNramp 3	*A. thaliana*	Fe, Cd, Mn	Vacuole	Roots/shoots	
AtNramp 4	*A. thaliana*	Fe, Mn		Roots/shoots	
OsNramp 1	*Oriza sativa*	Mn, Fe		Roots	Belouchi et al., 1995, 1997.
OsNramp 2	*O. sativa*	Mn		Leaves	
OsNramp 3	*O. sativa*	Mn		Roots/leaves	
LeNramp1	*Lycopersicum esculentum*	Fe		Roots	Bereczky et, al., 2003.
CDF					
ZAT (AtMTP1)	*A. thaliana*	Zn	Vesicular/vacuolar	All tissues	Van der Zaal et al., 1999.
TcZTP1	*T. caerulescens*	Zn	Intracellular membranes	Leaves, roots	Assuncão et al., 2001.

Continued...

Table 1. *continued...*

Name	Source	Proposed specificity	Cellular location	Main tissue expression	References
TgMIP1	*T. goesingense*	Cd, Co, Zn, Ni	Vacuole	Leaves	Persans et al., 2001.
ShMTP1	*Stylosanthes hamata*	Mn	Intracellular membranes	Roots, leaves	Delhaize et al., 2003.
PtdMTP1	*Populus trichocarpa x P. deltoids*	Zn	Vauolar membrane	Whole plant	Blaudez et al., 2003.
ZIP					
IRT1	*A. thaliana*	Fe, Zn, Mn, Cd	PM	Roots	Connoly et al., 2002.
IRT2	*A. thaliana*	Fe, Zn		Roots	Grotz and Guerinot, 2002.
OsIRT1	*O. sativa*	Fe		Roots	Bughio et al., 2002.
LeIRT1	*L. esculentum*	Fe (broad?)		Roots	Bereczky et al., 2003.
LeIRT2	*L. esculentum*	Broad?		Roots	
TcZNT1	*T. caerulescens*	Zn, Cd		Roots, shoots	Pence et al., 2000; Assuncão et al, 2001.
TcZNT2	*T. caerulescens*	-		Roots	
ZIPs1-3	*A. thaliana*	Zn		Roots	Grotz et al., 1998; Guerinot, 2000.
ZIP4	*A. thaliana*	Zn	Plastids	Roots, shoots	
GmZIP1	*Glycine max*	Zn	Peribacteroid membrane	Root nodules	Moreau et al., 2002.
Cation/H^+ antiporters					
CAX2	*A. thaliana*	Ca, Mn, Cd	Vacuole	Roots	Hirschi et al., 2000.
ABC transporters		Pc-Cd, GS-Cd			
ID17		Fe			Yamaguchi et al., 2002.
COPT1-5	*A. thaliana*	Cu		Leaves, stems flowers	Kampfenkel et al., 1995; Sancenón et, al., 2003 & 2004.
GNGC channels	*A. thaliana*	Ni, Pb	PM		Mäser et al., 2001.

Modified after Hall and Williams (2003)

4.2. Chaperones

In recent years a family of soluble metal receptor proteins, known as "metallochaperones", that are active in intercellular metal trafficking has emerged. These metal receptors are not detoxification proteins as they clearly function in a "chaperone-like" manner, but are involved in guiding and protecting the metal ion while facilitating its delivery to appropriate receptors.

Genetic studies in prokaryotic organisms and yeast have identified membrane-associated proteins that mediate either the uptake or export of copper from cells. Within these cells, small cytosolic proteins, designated as copper chaperones, have been identified. These bind copper ions and deliver them tospecific compartments and to copper requiring proteins (Peña et al., 1999). Three distinct copper trafficking pathways have been identified; these include delivery of copper to mitochondria, the *ATX1* pathway of copper delivery to Golgi, and copper delivery to cytosolic superoxide dismutase (see review by Halloran and Culotta, 2000).

A copper chaperone (CCH) and a responsive to antagonist 1 (RAN1), both identified in *A. thaliana*, were the first Cu delivery systems identified in plant cells (Himelblau and Amasino, 2000; Fig. 1; Table 1). The CCH, an Arabidopsis homolog to yeast copper chaperone antioxidant 1 (ATX1), is upregulated in senescing tissue (Himelblau et al., 1998), and it is involved in long-distance mobilization of Cu from senescing tissues via phloem (Mira et al., 2001). While the RAN1, an Arabidopsis homolog to yeast CCC2, involved in Cu trafficking, is involved in Cu delivery to ethylene receptors in post-Golgi vesicles (Hirayama et al., 1999; Fig. 1; Table 1).

Orthologues of the three copper chaperones characterized in yeast, ATX1, CCS and COX17, have been found in *A. thaliana* (Wintz and Vulpe, 2002). Genome analysis of Arabidopsis has revealed that *CCH* belongs to a family consisting of 31 genes coding for proteins that possess heavy metal associated (HMA) domains similar to the ATX1 HMA domain. Analysis of ATX1 homologues has suggested that these homologues play a variety of roles in intracellular and intercellular trafficking of Cu, and possibly of other metals, including transport of metals into chloroplast, detoxification, export of metals from cells, and possibly regulation of gene expression.

A plant homologue of CCS, the yeast chaperone to Cu/Zn superoxide dismutase, has been identified in tomato (Zhu et al., 2000) and Arabidopsis (Wintz and Vulpe, 2002). Wintz and Vulpe (2002) have concluded that the encoded protein has a potential chloroplast-targeting sequence indicating that it is a chloroplast protein, and it is most likely involved in maturation of the chloroplast Cu/Zn superoxide dismutase apoprotein. They have also identified two Arabidopsis homologues of COX17, including *AtCOX17-1* and *AtCOX17-2*, localized on chromosomes 3 and 1, respectively. The two COX17 proteins are predominantly expressed in roots, thus suggesting that their function may not be restricted to copper delivery to mitochondria, but that they may be also involved in copper transport in roots.

In plants, different members of the Cu chaperone family have been identified and characterized, including an Arabidopsis *CCH* (Himelblau et al., 1998) and *AtCOX17* (Balandin and Castresana, 2000), and a tomato *LYS7* (Zhu et al., 2000). Beyond the *CCH* gene (Mira et al., 2001), there is no information available on the expression of *COX17* and *CCS* (Wintz and Vulpe, 2002) genes in different plant organs and tissues and/or during plant development. However, Trinidade et al. (2003) have isolated and characterized a potato Cu chaperone gene *StCCS*, coding for the Cu/Zn superoxide dismutase (Sodp). Furthermore, they analyzed its expression throughout various tissues of a potato plant, and during tuberization.

4.3. Transporters

4.3.1. ABC – Type Family

The ATP binding cassette (ABC) protein superfamily is the largest membrane protein family known in both prokaryotes and eukaryotes, including microbes, plants, and animals. Members of this superfamily catalyze the MgATP-energized transport of a broad range of substrates across biological membranes. Extensive reviews have been written on plant ABC transporters (Theodoulou, 2000), and a complete inventory of ABC proteins in Arabidopsis have been compiled (Sanchez-Fernandez et al., 2001).

Isolation of *IDI7*, an Fe-deficiency-induced cDNA, from roots of barley was reported (Yamaguchi et al., 2002). Phylogenetic analysis revealed that IDI7 was closely related to the half-type ABC protein subfamily, which included mammalian transporters associated with antigen processing (TAPs). IDI7 and its orthologues seemed to comprise a new class of ABC transporters located in the tonoplast of higher plants. Accumulation of IDI7 mRNA was much lower under higher concentrations of heavy metals than under Fe-deficiency conditions, thus suggesting its lack of involvement in the sequestration of metal ions into vacuoles.

Shikanai et al. (2003) have characterized six Arabidopsis mutants defective in the *PAA1* gene which codes for a member of the metal-transporting P-type ATPase family with a functional N-terminal chloroplast transit peptide. PAA1 is a critical component of a Cu transport system in chloroplasts responsible for cofactor delivery to the plastocyanin and the Cu/ZnSOD. It has been reported that *paa1* mutants exhibit high-chlorophyll-fluorescence phenotypes due to impairment of the photosynthetic electron transport possibly ascribed to decreased levels of holoplastocyanin. Chloroplastic Cu/ZnSOD activity is also reduced in *paa1* mutants, thus suggesting that PAA1 mediates Cu transfer across the plastid envelope (Fig. 1).

Arababopsis thaliana has eight genes encoding members of the type 1_B heavy metal–transporting subfamily of the P-type ATPases (Hussain et al., 2004) (Table 1). Three of these transporters, HMA2, HMA3, and HMA4, are closely related to each other and have high sequence similarity to the divalent heavy metal cation transporters

of prokaryotes. Arabidopsis mutants for each of these transporters have been identified and characterized, and their roles in metal homeostasis have been determined. While individual mutants exhibited no apparent phenotype, *hma2 hma4* double mutants had a nutritional deficiency phenotype that could be compensated for by increasing the level of Zn in the growth medium, but not that of either Cu or Co. Levels of Zn, but not of other essential elements decreased in shoots of an *hma2 hma4* double mutant and to a lesser extent of an *hma4* single mutant when compared to wild type. Together, these observations indicate primary roles for HMA2 and HMA4 in essential Zn homeostasis. *HMA2promoter*- and *HMA4promoter*-reporter gene constructs revealed that *HMA2* and *HMA4* are predominantly expressed in vascular tissues of roots, stems, and leaves, and HMA2 appears to be localized to the plasma membrane. These observations are consistent with the roles proposed for HMA2 and HMA4 in Zn translocation. Both *hma2* and *hma4* mutations confer increased sensitivity to Cd in a phytochelatin-deficient mutant background, thus suggesting that they may also influence Cd detoxification.

The multidrug-resistance-associated protein (MRP) is a large subfamily of ABC transporters. Otherwise known as GS-X pumps, glutathione-conjugates, or multispecific organic anion Mg^{2+}-ATPases, MRPs are reported to participate in the transport of both exogenous and endogenous amphipathic anions as well as glutathionated compounds from the cytosol into the vacuole (Rea et al., 1998). AtMRPs, subfamily of Arabidopsis ABC transporters, are plant homologues of multi-drug-associated proteins. Their involvement in the transport of a wide variety of substances into the vacuoles of plant cells in higher plants has been proposed (Rea et al., 1998; Rea, 1999). When the role of AtMRPs in cadmium vacuolar sequestration has been investigated, expressed putative sequences coding for AtMRPs have been observed in roots and shoots of plants at different levels (Bovet et al., 2003). In 4-week-old Arabidopsis seedlings, transcript levels of four AtMRPs are up-regulated following Cd treatment, and these are exclusively observed in the roots. Interestingly, increases in transcript levels of AtMRP3 are most predominant. In young plantlets, a higher portion of Cd^{2+} is translocated to aerial parts compared with adult plants. Consequently, *AtMRP3* transcript levels increase in both roots and shoots of young plants suggesting that 7-day-old seedlings do not exhibit such a strict root-to-shoot barrier as 4-week-old plants. Expression analysis for glutathione and phytochelatin synthesis in mutant lines, as well as their subjection to compounds producing oxidative stress have indicated that induction of *AtMRP3* is likely due to the heavy metal itself.

Sancenón et al. (2004) first reported on the physiological function of copper transport in *A. thaliana*. Studies of expression pattern of *COPT1* in transgenic Arabidopsis plants overexpressing a reporter gene under the control of the *COPT1* promoter revealed its presence in embryos, trichomes, stomata, pollen, and root-tips. Moreover, investigating the involvement of COPT1 in copper acquisition in *CaMV35S::COPT1* antisense transgenic plants confirmed its role in Cu uptake. Interestingly, *COPT1* antisense plants also exhibited both increased root length, which was

completely and specifically reversed by adding copper, and increased sensitivity to growth inhibition by the copper-specific chelator bathocuproine disulfonic acid.

4.3.2. CDF – Type Family

Cation diffusion facilitator (CDF) proteins have been recently discovered, and these belong to a family of cation efflux transporters that likely play essential roles in metal homeostasis and tolerance. CDFs have six transmembrane domains, long cytoplasmic C-terminal tail domain, and histidine-rich region (Guerinot, 2000). Major substrates for these mechanistically poorly understood transporters are Zn^{2+} and Cd^{2+}. CDFs have been reported to confer tolerance for Zn^{2+}, Co^{2+}, or Cd^{2+} ions in bacteria (Nies 1992), yeast (Clemens et al., 2002; Borrelly et al., 2002), animals and plants (Blaudez et al., 2003).

Identification, characterization, and localization of PtdMTP1, a member of the CDF family in the hybrid poplar *Populus trichocarpa* x *Populus deltoids,* have been described by Blaudez et al. (2003). However, *PtdMTP1* is constitutively and ubiquitously expressed at low levels. Heterologous expression of *PtMTP1* in yeast has demonstrated its ability to complement the hypersensitivity of mutant strains to Zn, but not to other metals, such as Cd, Co, Mn, and Ni. In both yeast and in plant cells, *PtdMTP1* fused to a green fluorescent protein (GFP) was localized to the vacuolar membrane. This is consistent with its role in zinc sequestration. In fact, overexpression of PtdMTP1 in Arabidopsis has conferred Zn tolerance. When expressed in yeast and Arabidopsis, *PtdMTP1* forms homooligomers, a novel feature of CDF members. Oligomer formation is disrupted by reducing agents, thus indicating a possible disulfide bridge formation.

The yeast *Saccharomyces cerevisiae ShMTP1* encodes membrane-bound proteins of the CDF family involved in Mn^{2+} tolerance. When expressed in Arabidopsis, it conferred Mn^{2+} tolerance through internal sequestration (Delhaize et al., 2003). The ShMTP1 protein fused to a GFP was localized to the tonoplast of Arabidopsis cells, but it was localized in the endoplasmic reticulum of yeast.

4.3.3. Other Transporters

Among other transporters that have been identified so far, nine oligotransporter (OPT) orthologs (*AtOPT1* to *AtOPT9*) were identified in Arabidopsis (Koh et al., 2002). These had distinct tissue-specific expression patterns suggesting different functional roles. Complementary expression studies in yeast, *S. cerevisiae*, provided new evidence for presence of multiple peptide transporter systems in Arabidopsis. This also suggested important physiological roles for those small peptides in plants.

5. HYPERACCUMULATORS

Hyperaccumulation or hypertolerance refers to the plant's ability to grow on soils enriched in heavy metals such as Zn, Ni, Cu, Cd, or Pb, and it is usually metal-specific (Verkleij and Schat, 1990). A limited number of plant species, ~400 (Baker et al., 2000), have been classified as heavy metal accumulator plants. Despite recent advances (Assunção et al., 2001; Clemens, 2001; Macnair, 2002), mechanisms underlying metal hyperaccumulation are still largely unknown. Analysis of 20 *Brassicaceae* accessions collected from a wide geographical area has been initiated to identify potential metal hyperaccumulating species for use in large-scale genomic efforts to uncover genes involved in metal hyperaccumulation (Peer et al., 2003). Thus far, the first phase has revealed that the accession *T. caerulescenes* Félix de Pallières shows promise as a model hyperaccumulator. *T. caerulescens* is one of the relatively well-studied metal hyperaccumulators that grows on different metalliferous soil types, such as calamine and serpentine, as well as on nonmetalliferous soils. By comparing plant populations growing on contrasting soil types, it has been revealed that high–level tolerances are metal-specific and confined to those metals that are enriched at toxic levels in soils of a particular plant population site (Assunção et al., 2003).

Among others, *A. halerii* ssp. *halleri* (accession Langelsheim) is a naturally selected Zn- and Cd-tolerant Zn hyperaccumulator. This species differs strikingly from its close relative *A. thaliana* by specifically accumulating Zn in aboveground tissues. Recently, Becher et al. (2004) have used *A. thaliana* GeneChips to identify genes potentially involved in either cellular metal uptake or detoxification in shoots of *A. halerii*. Based on transcript levels, Zn tolerance in *A. halleri* involves high constitutive expression of metal homeostasis genes in shoots to accommodate for higher basal levels of Zn accumulation, and possibly to accommodate for sudden increases in Zn influx into shoots. Identified candidate genes coded for proteins closely-related to the following *A. thaliana* proteins, *At*ZIP6, a putative cellular Zn uptake system and a member of the zinc-regulated transporter (ZRT)-iron regulated transporter (IRT)-like protein (ZIP)-family of metal transporters, the putative P-type metal ATPase *At*HMA3, the cation diffusion facilitator ZAT/*At*CDF1, and the nicotianamine synthase *At*NAS3.

6. PHYTOREMEDIATION

Phytoremediation is a process whereby capabilities of green plants to take up and accumulate heavy metals are exploited for purposes of environmental clean-up. Most naturally-occurring hyperaccumulating plants have low biomass, and are restricted in their growth to certain areas. Therefore, the tools of molecular biology are utilized to implement all mechanisms involved in heavy metal resistance and accumulation in order to develop an environmentally friendly clean-up system for contaminated soils.

Recently many transgenic plants with increased heavy metal resistance and uptake of heavy metals have been developed solely for purposes of phytoremediation (Table 2).

Generally, there are two main approaches for phytoremediation: 1) developing transgenic plants capable of phytoextraction, and 2) identifying and/or developing plants capable of surviving on contaminated soils.

Rugh et al. (1997) expressed a bacterial mercuric reductase gene (*merA*) in yellow poplar and reported higher elemental mercury release from soil at approximately 10 times the rate of that of untransformed plants. More recently, phytoremediation of organomercurial compounds via chloroplast engineering has been reported (Ruiz et al., 2003). Genes coding for mercuric ion reductase (*mer A*) and organomercurial lyase (*merB*) were integrated into tobacco (*Nicotiana tabacum*) chloroplast genomes, and transplastomic were found to be substantially more resistant than wild-type plants to the highly toxic organomercurial compound phenylmercuric acetate. This novel approach might have applications to other metals as well.

In order to study the effects of *MerA* and *MerB* genes in plants, Pilon-Smits and Pilon (2000) developed *MerA* and *MerB* double-transgenic plants and evaluated their tolerance to organic mercury in comparison to their wild-type and *MerA*/*MerB* siblings. It was observed that double-transgenic plants showed the highest tolerance to organic mercury as they converted organic mercury to elemental mercury which was then released from the plant through volatilization.

In fact, volatilization of selenium in *Brassica juncea* plants was enhanced by overexpression of cystathionine-gamma-synthase, an enzyme that catalyzes the first step in the conversion of Se-cysteine to volatile dimethylselenide (van Huysen et al., 2003). Transgenic Indian mustard [*Brassica juncea* (L.) Czern.] plants overproducing gamma-glutamylcysteine synthase (ECS), gluthatione synthase (GS), or adenosine triphosphate sulfurylase (APS) were evaluated for phytoremediation of metal-contaminated mine tailings (Bennett et al., 2003). The ECS and GS transgenics accumulated significantly more metal in their shoots than wild-type plants, and significantly removed more metal from the soil. This was the first field study to demonstrate enhanced phytoextraction potential of transgenic plants using polluted environmental soil.

In order to reduce heavy metals in the food chain, plants that transfer lower amounts of heavy metals to shoots are critically important. The possibility of using a bacterial transporter gene to reduce heavy metal content in shoots has been reported (Lee et al., 2003). *E. coli ZntA* gene, which codes for a Pb (II) / Cd (II) / Zn (II) pump, has been used to transform Arabidopsis. Transgenic plants have shown improved resistance to Pb (II) and Cd (II), but with lowered contents of Pb and Cd in aerial parts of the plant.

Although several advances have been made in understanding plant's capability to either survive on and/or accumulate heavy metals in various tissues, additional knowledge is yet to be discovered. Pursuing further understanding of the fundamental mechanisms involved in hyperaccumulation processes that naturally occur in metal hyperaccumulating plants should allow for the development of plants that are more ideally suited for phytoremediation of metal contaminated soils. Although it is proposed that genetic hypertolerance is controlled by a small number of genes (Macnair 1993), molecular mechanisms accounting for hypertolerance remain poorly understood.

Table 2. *Genes introduced into plants and the effects of their expression on trace elements tolerance, accumulation or volatilization. Relative values refer to control plants not expressing the transgene. A, Accumulation in the shoot; GSH, gluthatione; MT, metallothionein; PC, phytochelatin synthase; T, Tolerance; and V, volatilazation*

Gene	Product	Source	Target	Effect	References
merA	Hg(II) reductase	Gram-Negative bacteria	*Liriodendron tulipifera*	T50 µM $HgCl_2$; 500 mg $HgCl_2$ Kg^{-1}	Rugh et al., 1998.
			Nicotiana tabacum	V Hg-volatilisation rate increased ten-fold	Heaton et al., 1998.
merA *merB*	Hg(II) reductase Organomercurial lyase	Gram-negative bacteria	*A. thaliana*	T 10 µM CH_3HgCl(>40-fold) V Up to 59 pg Hg(0)mg^{-1} fresh biomass min^{-1}	Bizili et al., 2000.
merA *merB*	Hg(II) reductase Organomercurial lyase	Gram-negative bactereia	*N. tabacum*	T 400 µM Phenyl nercuric acetate(PMA)	Ruiz et al., 2003.
AtPCSI	PC	*A. thaliana*	*A. thaliana*	T More sensitive to Cd	Lee et al., 2003a.
APSI	ATP sulfurylase	*A. thaliana*	*Brassica juncea*	A Two-fold increase in Se concentrations T As(III), As(V), Cd, Cu, Hg, Zn	Pilon-Smits et al., 1999.
				A Seedlings2.5-fold more As(III), As(V), Hg, Mo, Pb, V Mature plants 2.5-fold more Cd, Cu, Mo, V, W	Wangeline et al., 2004.
MT-I	M T	mouse	*N. tabacum*	T 200 µM $CdCl_2$ (20-fold)	Misra and Gedamu 1989; Pan et al., 1994 a, b.
CUPI	M T	*Saccharomyces cerevisiae*	*B. oleracea*	T 400 µM $CdCl_2$ (approx. 16-fold)	Hasegawa et al., 1997.

Continued...

Table 2. *Continued...*

Gene	Product	Source	Target	Effect	References
CGS	Cystathione-gamma-synthase	*A. thaliana*	*B. juncea*	V Se-volatilisation rate increased 2-3-fold	Van Huysen et al., 2003.
CSase	Cysteine synthase	*Spinach*	*N. tabacum*	T Increased tolerance to SO_3^{2-} T Increased tolerance to Cd, Se and Ni A 2.5 fold increase in shoot Cd accumulation	Noji et al., 2001; Kawashima et al., 2004.
gsh2	GSH synthase	*Escherichia coli*	*B. juncea*	A Cd concentrations 125% A 1.5-fold more Cd and 1.5-2.5-fold more Zn	Zhu et al., 1999a; Bennett et al., 2003.
gsh1	γ-Glu-Cys synthase	*E. coli*	*B. juncea*	A Cd concentrations 190% A. 1.5-fold more Cd; 1.5-2.5-fold more Zn; 2.4-3-fold more Cr, Cu, Pb	Zhu et al., 1999b; Bennett et al., 2003.
gsh1	γ-Glu-Cys synthase	*E- coli*	*Populus tremula x P. Alba*	A increased Cd tissue accumulation, no effect on Cd tolerance	Arisi et al., 2001.
NtCBP4	Cation channel	*N. tabacum*	*N. tabacum*	T 250 µM $NiCl_2$ (2.5-fold), Pb sensitive A Pb concentrations 200%	Arazi et al., 1999.
SL	Se-Cys lyase	mouse	*A. thaliana*	A Up to 1.5 fold increase in Se concentrations T Enhanced tolerance	Pilon et al., 2003.
TaPCSI	PC	*T. aesitivum*	*N. glauca*	T lmM $Pb(NO_3)_2$ and 30 µM $CdCl_2$ A Two-fold Pb increase	Gisbert et al., 2003.
ZATI	Zn transporter	*A. thaliana*	*A. thaliana*	T Slight increase	Van der Zaal et al., 1999.
ZntA	Pb(II)/Cd(II)Zn(II) transporter	*E. cole*	*A. thaliana*	T 700 µM Pb(II) and 70 µM Cd(II) V 2-3-fold increase for Pb and 1-2-fold for Cd	Lee et al., 2003.

Modified after Krämer and Chardonnens (2001)

7. CONCLUSION

The success of phytoremediation will require an improved understanding of the biological processes involved in metal acquisition, movement across plant cells within a plant, shoot accumulation, and metal detoxification in both metal hyperaccumulator and nonaccumulator plants. Once our knowledge of these intricate processes is further enhanced, we will be able to fully take advantage of plants for phytoremediation of numerous and vast areas of agricultural land and/or natural habitats.

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CHAPTER 9

METABOLIC ENGINEERING FOR STRESS TOLERANCE

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1. INTRODUCTION

1.1. The Need for Metabolic Engineering for Stress Tolerance in Crops

Abiotic stresses, such as drought, salinity, freezing and high temperature are important limiting factors for crop yield (Boyer, 1982). Stress can be defined as an influence that is outside the normal range of homeostatic control (Lerner, 1999). It has been estimated that annually about 42% of the crop productivity is lost owing to various biotic and abiotic stress factors (Oerke et.al., 1994). Additionally, some of the stress factors are increasingly troubling to our environment. More irrigated lands are becoming salinized. Only 35 % of the world's potential arable land is currently used, and furthermore, almost 25% of the total irrigated acreage in the world suffers from high salinity (Flowers and Yeo, 1995). It is therefore imperative to breed cultivars of major crops exhibiting tolerance to various stress factors, which ultimately could lead to increased agricultural production to guarantee long-term food security and stable crop production (Bray et. al. 2000; Zhang et. al. 2000). With an expected world population of 12 billion by 2050, there is an urgent need to triple food productivity within a few decades (Vasil, 2003).

As immobile organisms, many plants have evolved remarkable adaptations for various environmental stress factors. Stress triggers a response in plants, which brings change in its metabolite profile, like formation of compatible solutes, antioxidants, phytoalexins, protein protectants and cryoprotectants often due to up-regulation of metabolic pathways (Bohnert and Shen, 1999). Stress may also be responsible for recession

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or complete shutting down of various cell functions and maintenances, such as cell division (Guy, 1999; Taiz and Zeigler, 2002). Plant breeders and geneticists have utilized natural variability for stress tolerance within germplasm collections for selecting cultivars with improved stress tolerance (Bolaños et. al., 1993; Ceccarelli et. al., 1991, Chang and Loresto, 1986). However, this approach is limited by the availability of variability within the germplasm and crossability of the species.

Metabolic engineering can be defined as the manipulation of cellular, enzymatic, regulatory and transport processes using recombinant-DNA technology for the purpose of enhancing specific product yield (Bailey, 1991). Development of methods to transfer genes across species has led to research aiming to produce stress-tolerant transgenic crops using metabolic engineering. Today, scientific community is dedicated to identify potential target genes for metabolic engineering of crops for enhanced biotic and abiotic stress tolerance. Advancement in revealing the human, yeast, *Arabidopsis* and bacterial genomes, and in addition, quickly growing bioinformatics databases are playing a major role in this goal.

Although the principles of rational metabolic engineering have been well developed in microorganisms, its application in plants has progressed at a slower pace. One of the major limitations has been finding out which one of a multistep pathway can be manipulated to achieve a desired stress tolerance phenotype. In many cases, the connections between a pathway (or its end product or a specific gene) and stress tolerance have not been well established. Therefore, most research in model species aims to provide that connection between a gene and a stress tolerance phenotype. For the application of metabolic engineering in major crops, thorough field-level assessments of stress tolerance are required. Such assessments have not been realized in many of the studies and this has been articulated in Flowers et. al. (1997). Despite the complexity and lack of field studies, the past two decades have seen some impressive progress especially on understanding how plants cope with and adapt to various environmental stress factors. The objective of this chapter is to highlight some of the modern approaches to engineer transgenic crops tolerant to abiotic stress factors.

Since the literature contains a large number of stress factors and a larger number of tolerance mechanisms, the scope of this chapter is restricted to two aspects. Firstly, generalities in applying metabolic engineering for plant stress tolerance will be outlined. Secondly, select recent, metabolic engineering studies on manipulating plants for known stress-related pathways and processes will be reviewed. In this section, three sets of studies will be considered: (a) pathway manipulations to increase metabolites for specific stress factors, (b) manipulations of transport processes and (c) improving stress tolerance of plants by modulating regulatory proteins.

2. TOOLS FOR METABOLIC ENGINEERING OF CROPS

2.1. Gene Transfer Technology

Worldwide, the area under transgenic crop cultivation was increased from 4.2 to 104.7 million acres between 1996 and 2000 (Cockburn, 2002). Currently, scientists are enriched

with tools to transform almost any crop species (Li et. al., 2001). However, the efficiency of gene transfer remains low for many important crops and needs to be improved. There is an increased availability of expression elements such as stress inducible promoters, vectors, selectable markers and data on the stability of recombinant proteins expressed in transgenic plants (Curtis and Grossniklaus, 2003; Fischer et. al., 2004; Herbers and Sonnewald, 1999; Penna et. al., 2002; Schunmann et. al., 2003). Now, progress has been made to insert a single gene or multiple genes in a single transformation event (gene stacking), allowing one to build a multi-step pathway (Chen et. al., 1998, van Bel et. al., 2001). Expression of transgenes in the plastids *via* chloroplast transformation (Daniell and Dhingra, 2002) is used for high level expression of genes. Some of the technical problems surrounding gene transfer in particular crops will eventually be overcome.

2.2. Wild Species to Understand Specific Stress Adaptations

Many wild plants have evolved interesting, and often unique and complex stress adaptations. Therefore, it is possible to identify unique pathways, transport processes and regulatory networks, not present in current model species, like *Arabidopsis thaliana* and rice. For this purpose there is a need to develop genomic and bioinformatic resources on highly stress-tolerant plants and other organisms. One approach is to use comparative genomics to identify genes implicated in stress tolerance. For example, the genomes of wild stress-tolerant relatives to *Arabidopsis* and *Oryza* can first be exploited for novel genes.

Based on physiological and molecular characterizations available in the literature, many plant species could be suggested to be models to study specific stress adaptations. These include but not limited to *Thellungiella halophila* (Volkov et. al., 2003), *Limonium latifolium* (Rathinasabapathi et. al., 2001; Raman and Rathinasabapathi, 2003), *Mesembryanthemum crystallinum* (Vera-Esterella et. al., 2004), *Atriplex hortensis* (Shen et. al., 2003), *Porteresia coarctata* (Majee et. al., 2004), *Tortula ruralis* (Wood et. al., 1999) and *Xerophyta viscosa* (Garve et. al., 2003). Depending upon the kind of gene mining strategy, the whole genome sequence may not be required for all the stress-tolerant models. Genomic research tools such as cDNA libraries, expressed sequence tag (EST) libraries, profiles of stress modulated genes and gene products using microarray and proteomics technologies are required for various stress-tolerant wild plants and other organisms.

2.3. Selection of Targets for Metabolic Engineering

Specific metabolites and their synthetic pathways have been implicated in stress tolerance in plants. This has been done over the years using comparative physiology between stress-tolerant and non-tolerant species. Metabolic engineering for stress tolerance can be achieved by amplifying constitutive concentration of antioxidants and assembly of compatible solutes in the plant tissues (Bohnert and Shen, 1999; Verpoorte

et. al., 2000). Current engineering approaches depend upon the transfer of one or several genes encoding either biochemical pathways or endpoints of signaling pathways. These gene products guard plants against abiotic stresses both in direct and indirect ways. Major components or targets for engineering stress tolerance in plants are listed in Table 1.

Even if an investigator knows that a particular pathway can be useful for manipulation, identifying the enzyme with the "most control" over the pathway flux is difficult. Early views on metabolic regulation considered that a small number of key "regulatory" enzymes were rate-limiting in controlling the flux through a multistep pathway. But this view has been proven wrong by more recent research (Fell, 1992) and the flux control through a pathway is often shared among the steps. One way to find the contribution of each step to metabolite flux is to use the metabolic control (MCA) analysis. MCA analyses require the determination of the metabolite flux through a pathway after perturbing a single enzyme-catalyzed step. Perturbations can be achieved using specific inhibitors, mutants or transgenic modifications (Fell, 1997). Computer modeling of metabolic pathways can greatly facilitate MCA analysis (Lee et. al., 2003).

Table 1. *Major metabolic and transport components for engineering abiotic stress tolerance in plants*

Components	*Mode of action*
Growth regulators	Modification in hormone homeostasis
Heat shock proteins	Prevention or reversal of protein unfolding
Ion/proton transporters	Elimination and sequestration of toxic ions from the cytoplasm and organelles; ion uptake and transport
Membrane fatty acid composition	Increase in membrane fluidity and chilling tolerance
Osmoprotectants	Osmotic adjustm ent, protection of proteins and membranes, scavenging of reactive oxygen
Reactive oxygen scavenger	Detoxification of reactive oxygen species
Signaling pathway	Ca^{2+}-sensors/phosphorylation intervened signal transduction
Transcription factors	Transcriptional activation or upregulation of specific structural genes
Water status	Stomatal behavior; regulation of aquaporin in tonoplast and plasma membrane

2.4. Network Rigidity and Potential for Negative Alterations of Primary Pathways

Many pathways implicated in stress tolerance are the ones that branch from primary metabolic pathways. It has been noted that branch points in metabolic pathways are "rigid" and can not be redirected easily. In certain instances, redirection of the substrate to a new product has resulted in negative phenotypes because the substrate is also required by the plant for a primary function. In other instances, even if a pathway has been installed in a new organism, metabolite accumulation is poor due to non-availability, poor transport or low concentration or flux of the substrate. Metabolite accumulation could also be poor due to increased degradation of the reaction product in the new host. While all the outcomes of a metabolic engineering intervention cannot be predicted, reiterative engineering steps can be used to overcome network rigidity problems.

2.5. Identification of Pathways and Genes: The Post-genomic Model

Perception of the stress, signal transduction, activation of transcription factors and expression of structural genes are the main steps in stress response (Krauss, 2001). Initially, plants may respond to stress perception by global response, and then by a more precise or adapted specific stress response (Genoud and Metraux, 1999; Bartels, 2001; Pieterse et al., 2001). Since the availability of whole genome sequence of *Arabidopsis thaliana*, identification of genes for use in metabolic engineering for stress tolerance has enormously improved. Figure 1 shows various steps in the identification, characterization and use of cloned genes for metabolic engineering for stress tolerance. Many high throughput techniques such as microarray analyses, proteomics and metabolomics are available to quickly identify a large number of transcripts, proteins or metabolites altered in response to stress. While these techniques, as they are applied most commonly, will not identify a causal relationship between a gene, (enzyme or metabolite) with stress tolerance, they provide valuable data to make testable hypotheses. Additional validation can come from mutants affected for their stress tolerance or correlative data from different genotypes. Availability of T-DNA insertion mutants in most of the genes in Arabidopsis, makes it possible to test the function of genes whose functions are currently unknown. Table 2 provides select examples of early rational metabolic engineering work where a stress tolerant phenotype has been achieved. While much progress has been made in engineering metabolic and transport traits, the genes involved in structural and developmental adaptations to stress have not been understood and used for engineering.

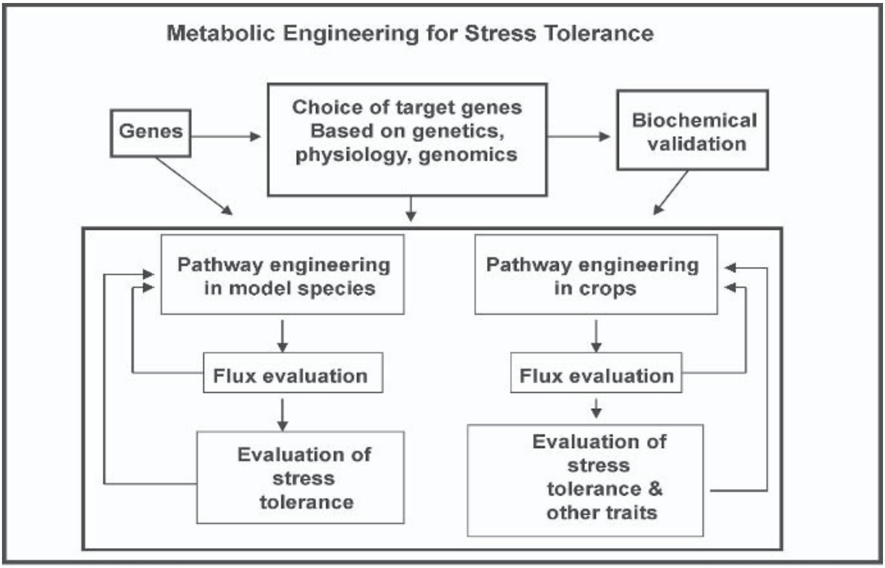

Figure 1 . *A model for metabolic engineering for stress tolerance*

3. PATHWAY ENGINEERING

3.1. Osmotic Stress

Organisms adapted to high salinity and drought, accumulate cytoplasmic solutes that act as protectants of membranes and proteins against damage by stress (also see Chapter 2 and 3). Small and very soluble molecules including proline and beta-alanine, quaternary ammonium compounds such as glycine betaine, proline betaine and beta-alanine betaine, sugar alcohols like mannitol, sorbitol and pinitol and, nonreducing sugars such as trehalose, function as osmoprotectants in various organisms (Rathinasabapathi, 2000 and 2002; Figure 2). These, jointly named as compatible solutes, help stressed plants by increasing the osmotic pressure within the plant and driving the gradient for water uptake under stress, therefore preventing water loss temporarily but facilitating to maintain permanent normal physiological ion balance (Yancey et. al. 1982). They also stabilize membranes and other macromolecular structures (Rhodes and Samaras, 1994).

Table 2. *Select "milestone" studies on metabolic engineering for stress tolerant plants*

Mechanism	*Stress tolerance*	*References*
Fatty acid unsaturation	Chilling	Murata et. al. (1992)
Antioxidation	Oxidative stress, drought	Gupta et. al. (1993a & 1993b)
Compatible solute synthesis	Salinity, cold and freezing	Tarczynski et. al. (1993)
Heat shock proteins and Chaperones	Heat	Lee et. al. (1995)
LEA proteins	Salinity, Drought	Xu et. al. (1996)
Glutathione and amino acid metabolism	Oxidative stress, drought, salinity	Roxas et. al. (1997)
Transcriptional control of stress pathways	Cold, drought	Jaglo-Ottosen et. al. (1998)
Sodium vacuolar transport	Salinity	Apse et. al (1999)

High concentrations of compatible solutes are non-toxic to the cells. So transgenic approaches can be used to facilitate their accumulation in crop plants for long-term osmotic stress tolerance (Chen and Murata, 2002; Serraj and Sinclair, 2002). Natural accumulation of osmoprotectants varies in plants i.e. 5-50 ìmol g^{-1} fresh weight (~6-60 mM on a plant water basis) and it often increases several-fold during exposure to osmotic stress (Rhodes and Hanson, 1993; Bohnert et. al., 1995). In plant cells, osmoprotectants are normally restricted to the cytosol, chloroplasts, and other cytoplasmic compartments.

Many crop plants do not naturally synthesize high levels of osmoprotectants. Therefore, it was hoped that crop stress tolerance could be improved by introducing one or many genes implicated in the synthesis of a specific osmoprotectant. Hence, metabolic engineering of synthetic pathways to osmoprotectants has been vigorously pursued during the last decade. Some examples for metabolic engineering of plants for osmoprotectant synthesis are listed in Table 3 and are discussed below.

3.1.1. Mannitol

The sugar alcohol mannitol is synthesized in numerous species of plants and its accumulation increases under dehydration stress (Patonnier et al., 1999). In celery, a natural accumulator of mannitol, its synthesis from mannose-6-phosphate is catalyzed by mannose-6-phosphate reductase (M6PR). Under stress, there is down-regulation of

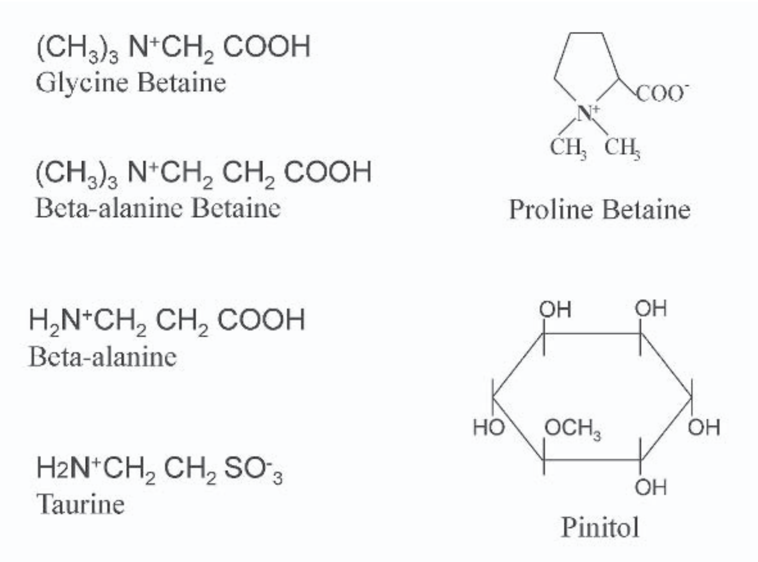

***Figure 2** . Structures of some osmoprotectants found in stress-tolerant organisms*

mannitol's utilization (Williamson et al., 1995). In the first demonstration of engineering for mannitol overproduction in plants, the gene for M6PR was not used. Instead, a bacterial gene for the reversible mannitol-1-phosphate dehydrogenase (mtl*D*), which catalyzes the conversion of fructose-6-phosphate to mannitol-1-phosphate was employed. This led to mannitol synthesis in transgenic tobacco, resulting in an osmotolerance phenotype against salinity (Tarczynski et. al., 1993; Karakas et. al., 1997).

In independent experiments, transgenic tobacco with increased mannitol in the chloroplasts was shown to exhibit increased resistance to methyl viologen-induced oxidative stress (Shen et. al., 1997). Transgenic wheat expressing the bacterial *mtlD* gene also exhibited tolerance to water stress and salinity (Abebe et. al., 2003), despite the lack of osmotically significant quantities of mannitol in these transgenic plants. This suggested that mannitol's effects are from protective mechanisms other than osmotic adjustment or *via* mannitol's osmoprotective role in the meristematic regions (Abebe et. al., 2003).

Arabidopsis, a non-mannitol producer, was transformed with the celery leaf M6PR gene under the control of the CaMV 35S promoter. Transgenic plants accumulated from 0.5 to 6 ìmoles g^{-1} fresh weight mannitol. Salt tolerance in transgenic plants was demonstrated in soil irrigated with 300 mM NaCl in the nutrient solution (Zhifang and Loescher, 2003). Pathways to other sugar alcohols have also been successfully installed in transgenic plants and stress tolerance phenotypes have been observed (Sheveleva et. al., 1997).

Table 3. *Some examples of genes utilized in pathway engineering to improve salinity and drought stress tolerance in plants*

Gene	*Gene action*	*Species*	*Phenotypic expression*	*References*
mt1D	Mannitol-1-phosphate dehydro genase (mannitol synthesis)	Tobacco, *Arabidopsis*	Increased plant height and fresh weight under salinity stress Increased germination under salinity stress	Tarczynski et al. (1993) ; Thomas et al. (1995)
m6pr	Mannose-6-phosphate reductase (mannitol synthesis)	*Arabidopsis*	Increased salt tolerance	Zhifang and Loescher (2003)
IMT1	Myo-inositol O-methyl transferase (D-ononitol synthesis)	Tobacco	Performed better under drought and salinity stress	Sheveleva et al. (1997)
otsA, otsB	Trehalose-6-phosphate synthase, Trehalose-6-phosphate phosphatase	Tobacco, Rice	Improved growth under stress	Goddjin and Van Dun (1999); Garg et al (2002)
TPS1	Trehalose synthesis	Tobacco	Stunted growth, improved drought tolerance	Romero et al (1997)
CodA	Choline oxidase (glycine betaine synthesis)	*Arabidopsis* Rice	Seedlings tolerant of salinity stress and increased germination under cold Increased tolerance to salinity and cold	Alia et al. (1998); Hayashi et al. (1997); Huang et al. (2000); Sakamoto et al. (1998)
GS2	Chloroplastic glutamine synthetase	Rice	Increased salinity resistance and chilling tolerance	Hoshida et al. (2000)
p5cs	Pyrroline carboxylate synthetase (proline synthesis)	Rice	Increased biomass production under drought and salinity stress	Zhu et al. (1998)

Certain plants that grow naturally in salt and drought conditions also manufacture methylated myo-inositol and its stereoisomers, like d-ononitol and pinitol (Ishitani et. al., 1997). Sheveleva et. al., (2000) showed that transgenic tobacco plants containing the myo-inositol-O-methyltransferase (*IMT1*) gene produced methylated inositol and the transgenic plants had higher tolerance for drought and salt stress than the vector controls.

3.1.2. Trehalose

Trehalose is a non-reducing disaccharide that functions as an osmoprotectant. It successfully stabilizes dehydrated enzymes and lipid membranes. Many bacteria and fungi accumulate trehalose. Only some plant species that can tolerate complete dehydration and spring back to life upon rehydration naturally accumulate trehalose (Goddijn and van Dun, 1999; Iturriaga et. al., 2000). Trehalose is synthesized in two steps from glucose-6-phosphate and uridine diphosphoglucose *via* trehalose-6-phosphate, in which the first step is mediated by trehalose phosphate synthase (TPS), and the second by trehalose-6-phosphate phosphatase (TPP) (Figure 3). In plants, trehalose-6-phosphate level is highly regulated by enzymes that either directly metabolizes it or by trehalase, which breaks down trehalose (Figure 3).

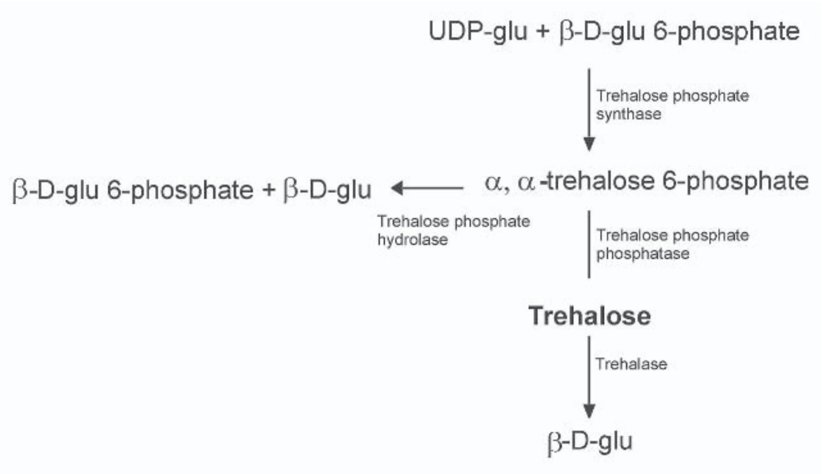

Figure 3. *Trehalose synthesis and metabolism*

Transgenic tobacco, expressing a bacterial trehalose-6-phosphate phosphatase (*OtsB*) gene showed better growth under drought stress (Pilon-Smits et. al., 1995). In addition, transgenic tobacco plants possessing yeast trehalose-6-phosphate synthetase gene (*TPS1*) exhibited improved drought tolerance (Romero et. al., 1997). Potato plants expressing genes for the trehalose synthesis showed unanticipated morphological changes and modification of structural carbohydrates (Yeo et. al., 2000). Therefore, a

trade-off between increased stress tolerance and unwanted physiological effects on plant growth needs to be considered.

In a recent example, Garg et. al., (2002) expressed bacterial genes (*otsA* and *otsB*) in rice using tissue-specific and stress-dependent promoters. Transgenic rice plants were not stunted and accumulated trehalose 3-10 times that of the non-transgenic controls and exhibited tolerance to salt, drought and low-temperature stress. Increased trehalose accumulation correlates with higher soluble carbohydrate levels and an elevated capacity for photosynthesis under both stress and non-stress conditions, consistent with a suggested role in modulating sugar sensing and carbohydrate metabolism. These findings demonstrate the feasibility of engineering rice for increased tolerance of abiotic stress and enhanced productivity through tissue-specific or stress-dependent overproduction of trehalose (Garg et. al., 2002). This made the authors suggest that stress inducible transgene expression is important in recovering trehalose-accumulating transgenics without deleterious effects. However, in a similar study where a bacterial gene encoding both trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase activities was expressed in rice under the control of a maize ubiquitin promoter (Jan et. al., 2002), stress tolerant transgenic rice with no growth abonormalities, was recovered, suggesting that rice may be more tolerant to trehalose pathway modification than dicots. It will be instructive therefore to engineer plants for novel osmoprotectants with and without stress inducible promoters and to use both monocots and dicots to evaluate the technology.

3.1.3. Fructans

Fructans are polyfructose molecules synthesized by many plants and bacteria mainly as a storage carbohydrate (Hendry, 1993; Pilon-Smits et. al., 1995). Fructans play an important role in root branching, thus helpful in increasing root surface area and water uptake by the plant. Transgenic plants overexpressing gene *sacB* exhibited higher capacity for osmotic adjustment. The additional gain in carbohydrate storage may direct deeper rooting and enhanced water uptake (Sharp et. al., 1996; Pilon-Smits et. al., 1995; Schellenbaum et. al., 1999).

3.1.4. Proline and Proline Betaines

Proline accumulation has been shown to be linked with drought and salinity stresses in plants (Delauney and Verma, 1990) and is caused by activation of proline biosynthesis and inhibition of proline degradation. Glutamate and ornithine are both precursors of proline. Proline is synthesized from glutamate *via* two intermediates, glutamic-Q-semialdehyde (GSA) and Ä-^{1}pyrroline-5-carboxylate (P5C), catalyzed by two enzymes, P5C synthase (P5CS) in the first step and P5C reductase (P5CR) in the final step. Overproduction of proline in transgenic tobacco (10-18 folds) (Kavi Kishor et. al., 1995), rice (Zhu et. al., 1998), wheat (Sawahel and Hassan 2002) and hybrid larch, *Laria*

leptoeuropaea (30 folds) (Gleeson et. al., 2005) with the Ä-[1]pyrroline-5-carboxylate synthetase (P5CS) gene improved root biomass and flower development under water and salt stress. Further work on transgenic soybean (De Ronde et. al. 2000) and *Arabidopsis* (Nanjo et. al., 1999a) plants transformed with the antisense gene for Ä-[1]pyrroline-5-carboxylate reductase and the gene for proline dehydrogenase (ProDH) (Nanjo et. al., 1999b) showed additional evidence that proline accumulation is positively correlated with stress tolerance in the plants.

The P5CS, Ä-[1]pyrroline-5-carboxylate synthase is subject to feedback inhibition by proline, thus is a rate-limiting enzyme in proline biosynthesis. Transgenic plants expressing a mutated form of the enzyme (P5CSF129A) whose feedback inhibition by proline was removed by site-directed mutagenesis accumulated about 2-fold more proline than the plants expressing *Vigna aconitifolia* wild-type P5CS (Hong et. al., 2000).

Transgenic lines overexpressing ornithine-d-aminotransferase (d-OAT) showed increase in d-OAT enzyme activity, resulted in higher proline production than the control plants and demonstrated a higher biomass as well as higher germination rate under osmotic stress conditions (Roosens et. al., 2002). Yonamine et. al., (2004) observed that overexpression of NtHAL3 genes isolated from *Saccharomyces cerevisae* conferred increased levels of proline biosynthesis in cultured tobacco cells and enhanced salt tolerance.

Proline is also a precursor for proline betaine and hydroxyproline betaines, excellent osmoprotectants found in certain members of the plant families Aizoaceae, Leguminosae, Rutaceae and Plumbaginaceae (Rathinasabapathi, 2002). The genes involved in proline betaine and hydroxyproline betaine synthesis routes have not yet been characterized and utilized in metabolic engineering.

3.1.5. Glycine Betaine

Glycine betaine is a significant compatible solute commonly distributed among plants. It is synthesized by a two-step oxidation of choline *via* betaine aldehyde. Choline oxidation in plants is catalyzed by choline monooxygnease while microbes employ either choline dehydrogenase or choline oxidase. Diverse plant species have variable capability to synthesize and accumulate glycine betaine. Relatively high levels of glycine betaine are found in plants like, spinach and barley as compared to plants that do not synthesize or accumulate it (e.g. *Arabidopsis* and tobacco). Metabolic engineering has been used to introduce genes for glycine betaine synthesis from both microorganisms and higher plants into betaine-deficient plants (Sakamoto and Murata, 2002). Numerous plant species have been engineered to synthesize elevated levels of glycine betaine. Many examples of recovery of stress tolerant transgenic plants have been reported (McNeil et. al., 1999, Chen and Murata, 2002). This technology has also been extended to several crops to recover stress-tolerant transgenic crops (Chen et. al., 2000; Huang et. al., 2000; Mohanty et. al., 2002; Prasad et. al., 2000a and 2000b., Sakamoto et. al., 1998; Shen et. al., 2002). In general, the transgenic plants engineered to produce glycine betaine made only small amounts of the osmoprotectant - much less than what

is observed in plants naturally resistant to stress (Hayashi et. al., 1997; Nuccio et. al., 2000; Sulpice et. al., 2003). Since accumulation of small quantities of glycine betaine appears to protect plants from a variety of stress factors, glycine betaine may have a role in stress tolerance by mechanisms other than it being an osmoticum.

Escherichia coli choline dehydrogenase has been introduced into tobacco alone and together with *E. coli* gene for betaine aldehyde dehydrogenase (Lilius et al., 1996; Holmstrom et al., 2000). Engineering the *codA* gene encoding choline oxidase from the soil bacterium *Arthrobacter globiformis* (Deshnium et. al. 1995) provided salt tolerance to transgenic to *Arabidopsis* (Hayashi et. al. 1997; Alia et. al., 1998), when grown on media containing 200 mM NaCl. Rice is a non-accumulator of glycine betaine (Ishitani et. al., 1993), but transgenic rice expressing *codA* gene exhibited normal growth at a faster rate than the wild type under salt stress (Sakamoto et. al., 1998). In addition, transformed *Brassica juncea* and Japanese persimmon (*Diospyros kaki*) overexpressing a gene for choline oxidase have shown tolerance to salt stress (Gao et. al., 2000; Prasad et. al., 2000a, 2000b). When a chimeric gene for betaine aldehyde dehydrogenase alone was expressed in the chloroplasts of carrot cells, some betaine was detected with a reported increase in salinity tolerance (Kumar et al., 2004). The nature of choline availability and choline oxidation step in this system is unclear because glycine betaine level has been shown to increase in plants that were engineered with the gene for betaine aldehyde dehydrogenase, the second step in the two step pathway to glycine betaine.

Glycine betaine synthesis in transgenic plants was limited by the availability of free choline because choline is essential for the synthesis of phosphatidyl choline of the membranes (Nuccio et. al., 2000). However, choline synthesis could now be engineered (McNeil et. al., 2001).

3.1.6. Beta-Alanine and Beta-Alanine Betaine

Beta-alanine is a non-protein amino acid and behaves as an osmoprotectant in microbial bioassays. In all plants, beta-alanine is one of the precursors for pantothenate, an essential vitamin (Figure 4). In most members of the stress tolerant plant family Plumbaginaceae, beta-alanine is the precursor for beta-alanine betaine, an excellent osmoprotectant (Hanson et al., 1991). We used *Limonium latifolium*, a member of the Plumbaginaceae as a model to investigate the synthetic pathway to beta-alanine betaine. Beta-alanine betaine synthesis is catalyzed by a trifunctional *N*-methyltransferase (Rathinasabapathi et. al., 2001). A full length cDNA for beta-alanine *N*-methyltransferase from *L. latifolium* was cloned and characterized recently (Raman and Rathinasabapathi, 2003). Experiments in our laboratory showed that in a variety of plant species, pantothenate synthesis was limited by d-pantoate and not by beta-alanine (Rathinasabapathi and Raman, 2005). Therefore, it should be possible to engineer the cDNA for beta-alanine *N*-methyltransferase in plants that do not naturally accumulate beta-alanine betaine, without significantly depleting pantothenate.

3.1.7. Polyamines

In rice and wheat, it has been found that polyamine accumulation affects drought and salinity tolerance of the plant. Transgenic plants engineered with a chimeric gene for *Datura* arginine decarboxylase showed increase in polyamine levels and ultimately, displayed 5-fold salt tolerance as compared to wild-type plants, and were capable of surviving and performing well for 20 d without water, whereas wild-type plants started deterioration if water was withheld for more than 4 d (Capell et. al., 2002; Capell, 2004).

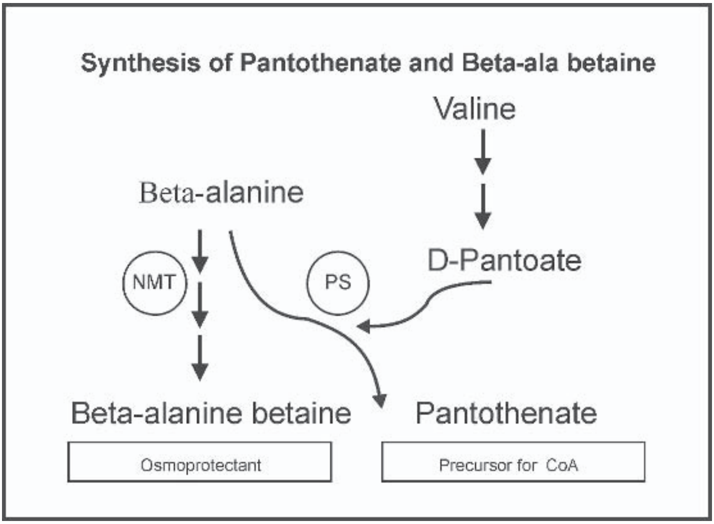

Figure 4. *In the members of the Plumbaginaceae, beta-alanine is shared between the synthesis of osmoprotectant beta-alanine betaine and pantothenate. NMT is a trifunctional beta-alanine N-methyltransferase (Raman and Rathinasabapathi, 2003) and PS is pantothenate synthetase (Genschel et. al., 1999), both presumably cytosolic enzymes*

3.1.8. Ectoine

Ectoine is an osmoprotectant found in *Halomonas elongata* and other halophilic bacteria. Aspartate semialdehyde is the precursor of ectoine (Ono et. al., 1999). Nakayama et. al., (2000) engineered tobacco with the three H. elongata genes (ectA, ectB, and ectC) encoding the enzymes responsible for ectoine synthesis. Transgenic cells were characterized by the production of low levels of ectoine (≤79 nmol g^{-1} fresh weight) and salinity tolerance.

3.1.9. Protein Kinase

There is a rapid increase in cytoplasmic Ca^{2+} levels in plant cells under various abiotic stress stimuli. Then, Ca^{2+} -dependent protein kinases (CPDK) bring about phosphorylation/ dephosphorylation of various proteins, which ultimately mediate Ca^{2+} influx signals (Sanders et. al., 1999). Rice plants engineered with altered levels of cold- and salt-inducible CDPK gene (OsCDPK7) showed high levels of salt and drought tolerance on rice plants (Saijo et. al., 2000).

3.1.10. Dehydrins

Plants also synthesize osmoprotectant proteins termed as dehydrins under drought and salinity stress. Overexpression of dehydrin HVA1 gene from barley (Xu et. al., 1996) and wheat (Cheng et. al., 2001, 2002) in transgenic rice resulted in resistance towards salt and water deficit stress.

3.2. Other Stresses

3.2.1. Anoxia/Hypoxia

Both limited supply of water or excessive flooding could cause oxygen deficiency in the roots. This deficiency triggers ethylene synthesis in the aerial parts of the plant and produces symptoms, such as chlorosis, senescence and ultimately death of plants. Certain plants however, are adapted to low oxygen by maintaining adequate supplies of energy and sugar to avoid self-poisoning and cytoplasmic acidosis (Stearns and Glick, 2003).

In general, plants produce ATP and NAD by means of glycolysis and fermentation rather than using Krebs cycle under oxygen limited conditions. A set of enzymes, such as alcohol dehyrogenase (ADH) and pyruvate decarboxylase (PDC) required for ethanolic and lactic acid fermentation is produced due to this anerobic switch (Andrews et al., 1994). Genes encoding ADH and PDC have been cloned and described (Bucher and Kuhlemeier, 1993; Umeda and Uchimiya, 1994; Hossain et. al., 1996, Quimio et. al., 2000).

Transgenic tobacco plants overexpressing a bacterial pyruvate decarboxylase gene showed 20-fold higher pyruvate decarboxylase activity in comparison to the wild-type (Bucher et. al., 1994, Bucher et. al., 1995, Tadege and Kuhlemeier, 1997, Tedege et. al., 1998). *Arabidopsis* genes encoding alcohol dehydrogenase, pyruvate decarboxylase and lactate dehydrogenase and alanine amino transferase were cloned and expressed in Arabidopsis plants (Dennis et. al., 2000). Transgenic cotton plants overexpressing cotton alcohol dehydrogenase gene displayed 10-30 fold increase in alcohol dehydrogenase activity and ethanol fermentation, whereas the cotton plants

expressing rice pyruvate decarboxylase had slightly more pyruvate decarboxylase activity than untransformed plants (Elllis et. al., 2000).

Under O_2 insufficiency conditions, the plant growth regulator ethylene is produced in the aerial plant parts. 1-Aminocyclopropane-1-carboxylic acid (ACC) is the precursor of ethylene. The enzyme ACC synthase is responsible for increase in ethylene synthesis in flooded plants. ACC is synthesized in the roots and converted to ethylene due to the action of ACC oxidase in the aerial parts (Grichko and Glick, 2001a). ACC oxidase requires oxygen for its activity. Transgenic tomato expressing antisense ACC synthase or ACC oxidase showed lower ethylene levels under root submergence (John, 1997; English et. al., 1995). Another enzyme ACC deaminase can draw away ACC from the ethylene synthesis pathway. Therefore, transgenic tomato plants expressing this catabolic enzyme displayed higher tolerance to flooding than non-transformed plants (Grichko and Glick, 2001b).

3.2.2. Heavy Metal Stress

Plants grown under acidic soils high in Al and Mn, saline soils high in Na and soils contaminated with As, Cu, Zn, Pb, Ni, and Cd due to mining, industrial effluents and other human activities or natural causes, show restricted growth and productivity (also see Chapter 7 and 8). Response of plants to heavy metal toxicity varies greatly, such as immobilization, exclusion, chelation and compartmentalization of the metal ions, and expression of general stress responses, like ethylene and stress proteins synthesis (Cobbett, 2000).

Certain toxic heavy metals can be removed from the soil or water using specific plants that can remove and concentrate the toxic metal in its harvestable parts. This technology, termed phytoremediation, is an environmentally friendly and cost effective method than engineering technologies for environmental remediation. Although naturally occurring heavy metal resistant plant species can be used for phytoremediation, characterization of genes involved in heavy metal resistance and hyperaccumulation, has led to the possibility for engineering plants for more efficient phytoremediation (Pilon-Smits and Pilon, 2002). Ideally a transgenic plant for phytoremediation will have the following characteristics: a) the plants will have the ability to grow at a fast rate under environments that need to be remediated, b) they will take up and concentrate the heavy metal into their harvestable biomass, c) they will not spread as uncontrolled weeds via vegetative means or pollen and d) they will pose little threat to animals and humans. Some examples for metabolic engineering for heavy metal stress tolerance are summarized in Table 4.

Table 4. *Examples for metabolic engineering for providing tolerance against heavy metal stress*

Metals	*Transgenic plant*	*Gene engineered*	*Source of gene*	*References*
Cadmium	Tobacco,	Metallothionein	Mus musculus	Pan et. al., 1994
Mercury	*Arabidopsis*	Mercury reductase (merA)	*Escherichia coli*	Rugh et. al., 1996
Aluminum	Tobacco	Citrate synthase	*Pseudomonas aeruginosa*	de la Fuente et. al., 1997
Cadmium	*B. oleracea*	Metallothionein	*Sacchromyces cerevisiae*	Hasegawa et. al., 1997
Mercury	*Liriodendron tulipifera*	Modified merA	*E. coli*	Rugh et. al., 1998
Cadmium	Tobacco	Metallothionein	*Nicotiana glutinosa*	Suh et. al., 1998
Lead, Nickel	Tobacco	Cation channel (NtCBP4)	*N. tabacum*	Arazi et. al., 1999
Mercury	*Arabidopsis*	MerB	*E. coli*	Bizily et. al., 1999
Selenium	*B. juncea*	ATP sulfurylase (APS1)	*Arabidopsis*	Pilon-Smits et. al., 1999
Zinc	*Arabidopsis*	Zn transporter ZAT1	*A. thaliana*	Van der Zaal et. al., 1999
Cadmium	*Brassica juncea*	Glutathione synthetase	*E. coli*	Zu et al., 1999a,b
Cadmium	Tobacco	Cysteine synthase gene	Rice	Harada et. al., 2001

3.2.3. Glutathione and Phytochelatins

The tripeptide glutathione is the most common thiol. It is a striking target for engineering stress tolerance in plants (Kunert and Foyer, 1993). Primarily, heavy metal sequestration by plants involves the formation of complexes with cysteine- rich peptides – glutathione derivatives called phytochelatins (PCs) (Figure 5). Cadmium forms a complex with phytochelatins in the cytosol and transported to the vacuole (Rauser, 1995)

and this is responsible for depletion of glutathione (Klapheck et. al., 1995; Schneider and Bergmann, 1995). Elevated γ-glutamylcysteine synthetase (γ-ECS) activity was shown to correlate with Cd resistance (Chen and Goldsborough, 1994). Poplar transformants overexpressing bacterial γ-ECS in the cytosol synthesized higher levels of glutathione (2-fold) even when exposed to different Cd concentrations (0–1000 $\mu g\ g^{-1}$ soil) for 14 d (Arisi et. al., 2000). In *B. juncea* transformed with E. coli gsh1 gene, encoding a γ-glutamylcysteine synthetase in the chloroplast, there was a three-fold increase in glutathione levels in comparison to non-transgenic control plants (Zhu et. al., 1999a). Also, expression of an *Escherichia coli* glutathione synthase gene (gsh2) in B. juncea resulted in a five-fold increase in root concentrations of glutathione, but only following exposure to cadmium (Zhu et. al., 1999b). A bacterial glutathione reductase (cp GR) expressed in *B. juncea* targeted to plastids exhibited 50 times higher activity of glutathione reductase and enhanced Cd tolerance than wild plants (Pilon-Smits et. al., 2000). The overexpression of a tobacco glutathione-S-transferase gene (parB) in Arabidopsis was reported to enhance Cu, Al, and Na tolerance (Ezaki et. al., 2000). Overexpression of enzymes γ-glutamylcysteine synthase (γ-ECS), glutathione synthetase (GS) and phytochelatin synthetase (PCS) in *Arabidopsis* significantly improved tolerance to As and Hg (Dhankher et. al., 2002). Harada et. al., (2001) also created transgenic plants with enhanced phytochelatin levels, through overexpression of cysteine synthase. The resulting transgenics demonstrated increased Cd tolerance, but lower Cd accumulation. The transgenic tobacco plants over-expressing cysteine synthase in the cytosol and chloroplasts, exhibited significantly more tolerance towards heavy metals such as Cd, Se and Ni (Kawashima et. al., 2004). Dhankher et. al., (2003) found that overexpression of bacterial arsenate reductase gene (arsC) provided Cd (II) resistance in tobacco and *Arabidopsis* plants.

3.2.4. Metallothioneins

Low molecular weight cysteine rich proteins called metallothioneins (MT) are synthesized in the cells in response to toxic heavy metals and help stabilize the concentration of the heavy metals in the cells (Hamer, 1985; Steffens, 1990). Transgenic tobacco plants expressing a metallothionein gene were able to grow in concentrations of up to 200 ìM CdSO4 (Liu et. al., 2000). Transformants of *B. oleracea* expressing the yeast metallothionein gene CUP1 tolerated 400 ìM Cd, whereas wild-type plants were unable to grow at concentrations above 25 ìM cadmium in a hydroponic medium (Hasegawa et. al., 1997).

3.2.5. Cation Diffusion Facilitators

Recently, a family of cation efflux transporters was discovered known as cation diffusion facilitator (CDF) proteins or metal tolerance proteins (MTP) that might play a vital

role in metal homeostasis and tolerance. *Arabidopsis* plants overexpressing ZAT1 or AtMTP1, encoding a putative zinc transporter in the CDF family of membrane transporters, accumulated higher zinc concentrations in their roots and also displayed better root growth in comparison to controls, when grown in a hydroponic media containing 200 ìM zinc. (Van der Zaal et. al., 1999; Maser et. al., 2001). Overexpression of PtdMTP1 (Populus trichocarpa x deltoids metal tolerance protein), in *Arabidopsis* conferred Zn tolerance (Blaudez et. al., 2003).

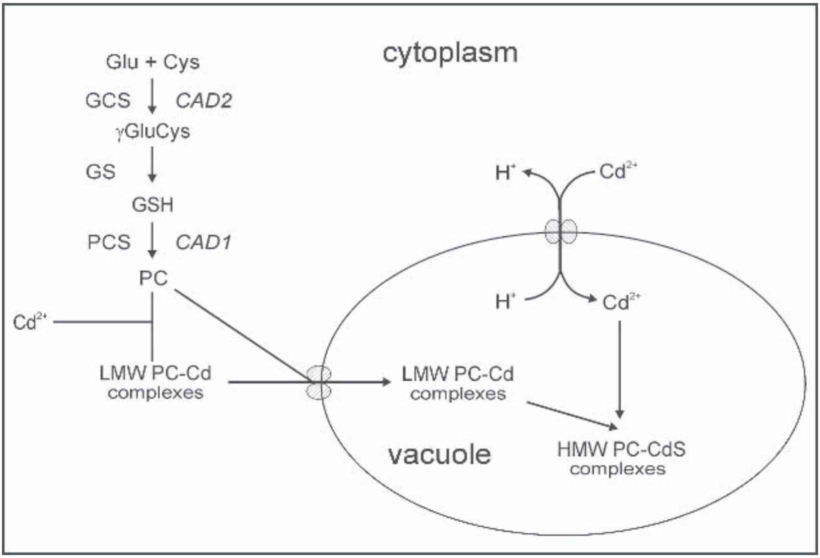

Figure. 5. *Glutathione mediated sequestration of Cadmium (Modified from Cobbett et al., 2000). Phytochelatin synthesis shown in the cytoplasm is a 3-step pathway. LMWPC-Cd refers to low molecular weight complex of PC and Cd and HMW PC-Cd shown in the vacuole refers to high molecular weight complex*

3.2.6. Citrate Synthesis and Other Metabolic Targets

Some plants have a capacity to tolerate Al toxicity by the process of exclusion of uptake from the root. These plants release organic acids such as citric acid, which chelates Al^{3+} outside the plasma membrane. Transgenic tobacco, papaya and *Arabidopsis* that overexpressed a citrate synthase gene (CSb) from *Pseudomonas aeruginosa* in their cytoplasm showed higher tolerance against Al toxicity (de la Fuente et. al., 1997; Koyama et. al., 2000; Lopez-Bucio et. al., 2000; Guerinot, 2001). In alfalfa, overexpression of two genes encoding enzymes phosphoenolpyruvate carboxylase (PEPC) and malate dehy-

drogenase (MDH) displayed higher synthesis of citric acid and Al tolerance (Tesfaye et al., 2001).

Transgenic *Arabidopsis* plants expressing MerA gene showed significantly enhanced tolerance to Hg (II) and volatilized elemental mercury (Rugh et. al., 1996). Also, transgenic MerB *Arabidopsis* plants were significantly more tolerant to methylmercury and other organomercurials (Bizily et. al., 1999). Transgenic tobacco and yellow poplar expressing both MerA and MerB genes also showed enhanced mercury tolerance (Rugh et. al., 2000).

Transgenic tobacco overexpressing NtCBP4 (*Nicotiana tabacum* calmodulin-binding protein) demonstrated enhanced tolerance to Ni^{2+} and hypersensitivity to Pb^{2+} due to reduced Ni^{2+} accumulation and Pb^{2+} accumulation respectively (Arazi et. al., 1999). Furthermore, expression of a truncated tobacco NtCBP4 channel in transgenic plants and disruption of the homologous *Arabidopsis* CNGC1 gene conferred improved tolerance to Pb^{2+} (Sunkar et. al., 2000). In an approach to enhance Se assimilation by plants, the plastidic *Arabidopsis* APS1 cDNA encoding ATP sulfurylase, was expressed in B. juncea under the control of a 35S promoter. Transgenic plants displayed a little increase in Se tolerance, and accumulated about 2-fold more Se in their shoots (Pilon-Smits et. al., 1999). Grichko et. al. (2000) found that the overexpression of 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase led to an enhanced accumulation of a variety of metals, as well as higher metal tolerance.

3.2.7. Arsenic Hyperaccumulation

Arsenic is a toxic carcinogen. In many areas of the world, the soil and ground water are contaminated with arsenic both due to natural and human activities. Current methods used to remediate arsenic contaminated water and soil are expensive. Recent research has uncovered the extraordinary ability of the Chinese brake fern (*Pteris vittata*) to hyperaccumulate arsenic (Ma et al. 2001), leading to phytoremediation technology to remediate arsenic contaminated environment.

Arsenic exists most commonly in two forms, As V and As III, the latter being more reactive and toxic to cells. To understand how the Chinese brake fern hyperaccumulates arsenic, the roots were exposed to As V in a nutrient solution medium and the total arsenic and the species of As were analyzed in the plants' fronds and roots. About 90% of the arsenic accumulated in the fern occurred in the form of As III and the accumulation was more in the shoots than in the roots (Tu et al., 2002; Figure 6). This shows that the fern has an efficient arsenic uptake system in its roots, and the fren's tissue reduces As V to As III and arsenic hyperaccumulation occurs in the fronds.

The mechanisms involved in arsenic hyperaccumulation in this fern are actively researched in many laboratories in the world including that of the authors. P.vittata genes involved in arsenic uptake, arsenate reduction, translocation, vacuolar transport

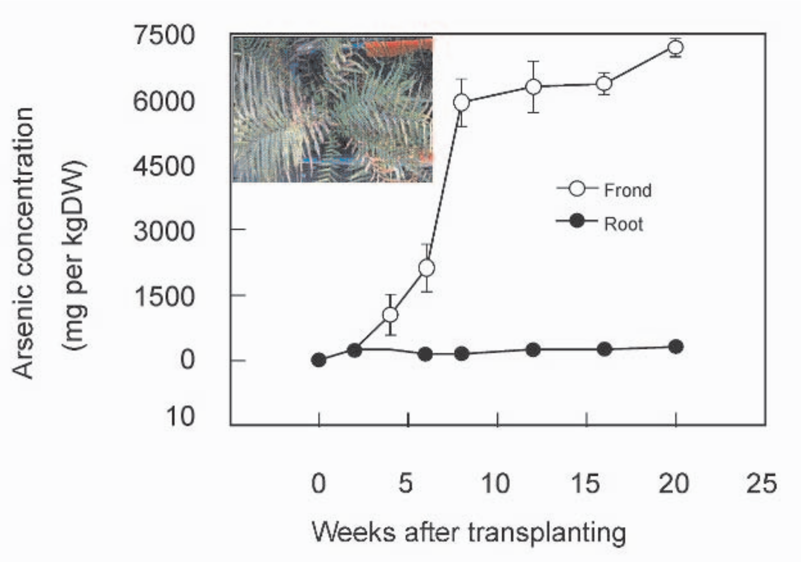

Figure 6. *Hyperaccumulation of arsenic in the shoots of Chinese brake fern Pteris vittata, when grown in a hydroponic nutrient solution containing 1000 ppm sodium arsenate (Adapted from Tu et. al., 2002). Inset is a picture of the fern*

and sequesteration will be useful in the future to engineer "remediation ready" transgenic plants for efficient phytoremediation of arsenic contaminated soil and ground water.

3.2.8. Oxidative Stress Tolerance

Even though, molecular oxygen is vital for the survival of living organisms, it offers a challenge to aerobic living beings through the synthesis of reactive oxygen species (ROS). Increased production of ROS is one of the facets of most environmental stress factors such as drought, salinity and heavy metal stress. Development of ROS is responsible for the creation of oxidative stress. Significant damage to the plants is caused by quick buildup of toxic products from reactions of ROS with lipids and proteins. Under oxidative stress, formation of lipid peroxides (chemically or enzymatically) causes cellular damage (Chia et. al., 1984; Dhindsa et. al., 1981). Antioxidative enzymes such as superoxide dismutase (*SOD*), ascorbate peroxidase (*APX*), glutathione peroxidase (*GPX*) and glutathione S-transferase (*GST*), and low-molecular weight antioxidants such as ascorbic acid and flavonoids are present in plants (Yu et. al., 1998; Yu and

Rengel, 1999). Specific role of these enzymes or small molecules are better understood by manipulating expression of the genes encoding antioxidant enzymes or by engineering synthetic pathways to antioxidants (Allen et. al., 1997). Since many of both enzymatic and non-enzymatic antioxidant systems are understood in plants, it must be possible to improve oxidative stress tolerance in crops *via* metabolic engineering. Some examples for such metabolic engineering attempts are listed in Table 5.

Transgenic tobacco, lucerne, potato and cotton overexpressing *SOD* in the chloroplasts showed higher tolerance to oxidative stress (Bowler et. al., 1991, Gupta et. al., 1993a, b, McKersie et. al., 1993, 1996, Perl et. al., 1993, Foyer et. al., 1994, Slooten et. al., 1995). Similar results were observed with overproduction of SOD in the mitochondria of lucerne (McKersie et. al., 1997) and in the cytosol of potato (Perl et. al., 1993). Transgenic tobacco engineered to overexpress MnSOD in chloroplast provided protection from Mn deficiency mediated oxidative stress (Yu et. al., 1999).

The tripeptide glutathione, the major cellular antioxidant, detoxifies the excess hydrogen peroxide generated during oxidative stress. Glutathione metabolism has therefore been manipulated to improve plant's oxidative stress tolerance (Noctor et. al., 1998). Transgenic plants overexpressing glutathione reductase had an increase in their reduced:oxidized glutathione ratio with enhanced tolerance to oxidative stress (Noctor et. al., 1998). Glutathione peroxidase is responsible for removal of unsaturated fatty acid hydroperoxides generated in cellular membranes during oxidative stress. Transgenic tobacco plants overexpressing *Chlamydomonas* glutathione peroxidase in the chloroplast or the cytosol, exhibited higher membrane integrity by increasing tolerance against oxidative stress (Yoshimura et. al., 2004).

3.2.9. Heat and Cold Stress

Temperate and subtropical plants are extremely vulnerable to high temperatures especially during early tillering, flower initiation, anthesis and grain filling stages, leading to considerable decrease in crop productivity. All tropical crops are exposed to high temperatures and can benefit by increasing their high temperature stress tolerance further (also see Chapter 4). However, this trait is particularly valuable in some of the world's most important food crops naturally adapted to cold weather. For example, improving the heat stress tolerance in winter cereals and potato can extend these crops to areas currently not suitable for them and increase their yield potential in some of the currently grown locations (Veilleux et. al., 1997; Maestri et. al., 2002). Despite our vast knowledge on how organisms respond to high temperature stress, little progress has been made in breeding crops for high temperature stress tolerance.

3.2.10. Heat Shock Proteins

In response to high temperature, all organisms including plants produce a set of proteins known as heat shock proteins (HSPs). Heat shock proteins (HSPs) have been

Table 5. *Metabolic engineering of genes encoding antioxidant enzymes to combat oxidative stress in plants*

Gene	*Gene action*	*Species*	*Phenotypic expression*	*References*
Sod	Cu/Zn superoxide dismutase	Tobacco, tomato	No protection seen against superoxide toxicity	Tepperman and Dunsmuir (1990)
	Mn superoxide dismutase	Tobacco	Reduced cellular damage under oxidative stress	Bowler et. al., (1991)
	Cu/Zn superoxide dismutase	Tobacco	Retained 90% photosynthesis under chilling and heat stress	Gupta et. al., (1993a)
	Mn superoxide dismutase	Alfalfa	Increased tolerance to freezing stress	McKersie et. al., (1993)
	Fe superoxide dismutase	Tobacco	Protected plants from ozone damage	Van Camp et. al., (1996)
	Mn superoxide dismutase	Alfalfa	Increased tolerance to water deficit	McKersie et. al., (1996)
Nt107	Glutathione S-transferase	Tobacco	Sustained growth under cold and salinity stress	Roxas et. al., (1997)
MsFer	Ferritin (iron storage)	Tobacco	Increased tolerance of oxidative damage caused by excess iron	Deak et. al., (1999)
Sod	Mn superoxide dismutase	Alfalfa	Increased winter survival	McKersie et. al., (1999)
	Mn superoxide dismutase	Tobacco	Increased tolerance to Mn deficiency	Yu et. al., (1999)
Apx3	Ascorbate peroxidase	Tobacco	Increased protection against oxidative stress	Wang et. al., (1999)
ParB	Glutathione S-transferase	*Arabidopsis*	Protects against Al toxicity and oxidative stress	Ezaki et. al., (2000)
GST/ GPX	Glutathione S-transferase	Tobacco	Increased stress tolerance with glutathione peroxidase	Roxas et. al., (2000)
Sod	Cu, MN, Fe, Zn-SOD	Alfalfa, rye grass	Increased winter hardiness	McKersie (2001)

implicated to have an adaptive role in plant's tolerance to heat stress (Vierling, 1991; Klueva et al., 2001). It is believed that HSPs exhibit chaperone functions by assisting other proteins in proper post-translational folding and maintaining them in a functional state. Transgenic expression of HSPs has increased the thermotolerance in many instances. A transgenic approach was successfully used for manipulating HSP expression and heat tolerance in *Arabidopsis* (Lee et. al., 1995; Lee and Schöffl, 1996; Prandl et. al., 1998; Malik et. al., 1999; Queitsch et. al., 2000; Sanmiya et. al., 2004) (Table 6).

Table 6. *Utilization of various heat shock protein families for development of thermotolerant transgenic plants*

Gene	*Gene action*	*Species*	*Phenotypic expression*	*References*
Hsp70	Heat-inducible antisense HSP70	*Arabidopsis*	Increased thermo tolerance in transgenic plants	Lee and Schoff (1996)
Hsp17.7	Heat shock protein	Carrot	Increased or decreased thermotolerance	Malik et. al., (1999)
Hsp101	Heat shock protein	*Arabidopsis*	Decreased thermotolerance in *Hsp101*-deficient (*hot1*) mutant	Hong and Vierling (2000)
Hsp101	Heat shock protein	*Arabidopsis*	Manipulated themotolerance in transgenic plants	Queitsch et. al., (2000)

Transgenic tobacco expressing class I cytosolic small HSP gene, *TLHS1* showed up to two times higher cotyledon opening rates in comparison to the transgenic tobacco seedlings carrying the *TLHS1* gene in antisense orientation at high temperature stresses of 40 °C and 45 °C for 1 to 4 h (Park and Hong, 2002). Expression of *Athsp101* and sHSP17.7 into the basmati rice resulted in higher survival and better growth performance in the recovery phase following the heat stress (Agarwal et. al., 2003; Murakami et. al., 2004). Overexpression of certain transcriptional regulators of HSP expression, like HSF1 and HSF3, triggered plants to constitutively express HSPs for higher basal thermotolerance (Lee et. al., 1995; Prändl et. al., 1998).

3.2.11. Amino Acids and Derivatives

Heat shock is also known to influence the level of certain free amino acids. Heat shock resulted in a significant increase of gamma-aminobutyric acid, beta-alanine, alanine, proline and tyrosine in cowpea cells (Mayer et. al., 1990). *Arabidopsis* plants engineered to overproduce glycine betaine, showed increased thermotolerance (Alia et. al., 1998), suggesting that nitrogenous osmoprotectants can have a role in cellular protection against heat stress. We have engineered tobacco plants to express bacterial aspartate decarboxylase, the enzyme catalyzing the decarboxylation of aspartate to beta-alanine. The transgenic plants expressing the bacterial gene contained significantly increased levels of beta-alanine and total free amino acids and exhibited a thermotolerant phenotype, when compared to control plants (Fouad and Rathinasabapathi, unpublished). Our results are consistent with the hypothesis that beta-alanine could have a role in thermotolerance. Further research is in progress to explore whether beta-alanine and other amino acid synthesis pathways could be potential avenues to improve crop tolerance to heat stress.

3.2.12. Fatty Acid Unsaturation

The cold sensitivity of the plant is closely linked with the degree of unsaturation of fatty acids in the membranes. Plants with elevated levels of cis-unsaturated fatty acids, like spinach and *Arabidopsis*, are resistant to cold, whereas species having only a small quantity, like squash are not. Many plant species produce high amounts of polyunsaturated fatty acids, especially 18:3 (linolenic acid) in membrane when exposed to low temperatures (Smolenska and Kuiper, 1977; Clarkson et. al., 1980; Kodama et. al., 1995). Following increase in polyunsaturated fatty acids, restriction of membrane permeability (Kuiper, 1974) and reduction in membrane-associated enzymes activities (Cronan and Gelmann, 1975) bring about an increase in membrane fluidity (Chapman, 1975). Glycerol-3-phosphate acyl transferase is an important determining factor for the level of phosphatidylglycerol fatty acid unsaturation. Tansgenic tobacco plants expressed a fair degree of cold tolerance following the manipulation of cholotoplastic enzyme glycerol-3-phosphate acyl transferase from squash and Arabidopsis (Murata et. al., 1992; Murata and Tasaka 1997; Sakamoto et. al., 2003). The ù-3 fatty acid desaturases are membrane-bound enzymes found in plastids (Mazliak, 1994) that catalyze the conversion of 18:2 (linoleic acid) to 18:3. The overexpression of the plastidal ù-3 fatty acid desaturase genes (*NtFAD7* and *NtFAD3*) in transgenic tobacco provided increased chilling tolerance (Kodama et al., 1994; Hamada et. al., 1996, 1998). Transformation of *Arabidopsis* with a codA gene encoding choline oxidase enhanced cold tolerance during germination and early growth (Alia et. al., 1998).

3.2.13. Cold Acclimation

Extended stay of cold-tolerant plants under cold temperature (below 5°C) makes them more freezing tolerant due to activation of cold acclimation response (COR) genes (also see Chapter 5). This process is generally termed as cold acclimation or cold hardening (Strand et. al., 2003). Artus et. al. (1996) demonstrated that constitutive expression of the cold regulated *Arabidopsis COR15a* gene affects both chloroplast and protoplast freezing tolerance. Kaye et. al (1998) made transgenic tobacco plants that constitutively expressed the spinach cold acclimation proteins (CAP85 and CAP160).

3.2.14. Carbohydrate Metabolism

Cold acclimation and winter survival in plants is strongly associated with the revival of photosynthesis at low temperature (Stitt and Hurry, 2002), and storage of soluble carbohydrates, like sucrose and raffinose (Olien and Clark, 1993). Transgenic *Arabidopsis* plants with overexpression of sucrose phosphate synthase (*sps*) showed improved photosynthesis and increased sucrose flux in comparison to both plants with antisense repression of either cytosolic fructose-1,6-bisphosphatase (*fbp*) or SPS at 5^0C (Strand et. al., 2003). Down-regulating a-galactosidase (*Lea-Gal* gene) in petunia using antisense technology resulted in an increase in freezing tolerance as α-galactosidase is responsible for the degradation of raffinose (Pennycooke et. al. 2003). Transgenic tobacco plants accumulating high levels of proline, fructans, or glycine betaine exhibited tolerance to cold temperature (Konstantinova et. al., 2002; Parvanova et. al., 2004). Overexpression of glutathione S-transferase/glutathione peroxidase resulted in plants tolerant to chilling stress (Roxas et. al., 1997). A novel plant NADPH-dependent aldose/aldehyde reductase, was identified in cultured bromegrass cells associated with the induction of freezing tolerance (Lee and Chen, 1993).

3.2.15. Shading Stress

Biomass production is highly dependent upon optimum supply of nutrients and efficient photosynthesis, whereas the harvest index and economic yield are governed by allocation of assimilates within the developing plant. Under crowding and shading conditions in a single as well as mixed cropping pattern, struggle for light energy calls up for shade avoidance syndrome manifested by quick growth, and extension of stem and petiole at the expense of leaves, storage and reproductive organs thus making plants vulnerable to lodging, diseases and insect pests, and ultimately, a lower harvest index (Morgan and Smith, 1976; Deregibus et. al., 1983; Rousseaux et. al., 1999; Smith, 2000). Light reflected from adjacent vegetation is rich in far-red (FR) and depleted in red (R) wavelengths due to its absorption by chlorophyll. This alteration in light quality is sensed by photoreceptors called phytochromes (Quail, 1998). Phytochromes present in two photo-interconvertible forms, an inactive, R-absorbing Pr-form and an active, FR

absorbing Pfr-form. R FR ratio (R:FR) of light incident upon the plant is responsible for setting up a dynamic equilibrium between the two forms (Smith and Holmes, 1977). In open canopies, a high R:FR favors Pfr-form, which suppress elongation, growth and flowering of the plant, resulting in a normal and healthy growth pattern (Whitelam and Devlin, 1997). On the other hand, low R:FR reflected from adjacent foliage drives phytochrome equilibrium in the direction of Pr, activating shade avoidance response (Smith, 1994; Delvin et. al., 2003). Changes in the R to FR ratio perceived by phytochromes initiate a number of responses such as increased stem extension growth. Stem elongation is blocked when phytochrome A (PhyA) is present at high levels. Therefore, transgenic approaches can manipulate phytochrome genes to disable responses to R to FR ratio so that a higher proportion of resources can be incorporated into harvestable material of crops (Smith, 1992). Transgenic tobacco ectopically expressing oat PHYA were indistinguishable from wild-type at the lowest plant density but became gradually shorter as the plant density increased and led to increase in harvest index. This demonstrated the suppression of shade avoidance response under high level of phyA expression (McCormac et. al., 1992; Schmitt et al., 1995; Robson et. al.; 1996; Shlumkov et. al., 2001). Phytochrome A overexpression also inhibited hypocotyl elongation in transgenic *Arabidopsis* (Boylan and Quail, 1991, Thiele et. al., 1999). Expression of the *Arabidopsis PHYB* (phytochrome B) gene can increase tuber yield by increasing photosynthesis and delayed leaf senescence at high plant densities in field-grown transgenic potato (Thiele et. al., 1999; Boccalandro et. al., 2003; Schittenhelm et. al., 2004).

Other strategies for increasing photosynthetic activity include modification of key photosynthetic enzymes, and conversion of C_3 photosynthetic pathway into C_4. An effort has been made to transform Rubisco and the enzymes of the Calvin cycle in tobacco (Miyagawa et. al., 2001; Whitney and Andrews, 2001). Transgenic rice plants were also produced expressing pyruvate orthophosphate dikinase and NADP-malic enzyme (Ku et. al., 1999). The maize gene encoding phosphoenolpyruvate carboxylase (PEPC) has been transferred into several C_3 crops, including potato (Ishimaru et. al., 1998) and rice (Matsuoka et. al., 1998; Ku et. al., 1999) in order to increase the overall level of C fixation.

3.2.16. UV-B Stress

Destabilization of ozone layer is resulting in an increase in the ultraviolet-B (UV-B: 280–320 nm) radiation reaching the earth's surface. This has increased our attention about potential effects of increased UV-B levels on plant growth and development. UV-B radiation affects plants by causing damage to DNA directly or indirectly through formation of free radicals (Kalbin et. al., 2001), photosystems, phytohormones, and membranes by peroxidation of unsaturated fatty acids (Mackerness 2000). A number of secondary metabolites such as flavonoids, tannins and lignins are increased at elevated levels of UV-B radiation. These metabolites screen UV-B and protect the cellular components against the UV-B damage (Hofmann et. al., 2000, Kondo and Kawashima 2000, Ryan et al., 2002),

In *Arabidopsis*, overexpression of bacterial *chyB* gene encoding β-carotene hydroxylase exhibited enhancement in photooxidative stress tolerance due to specific increase in xanthophyll (Davison et. al., 2002). Similarly, transgenic tobacco plants overexpressing a bacterial carotene hydroxylase gene responsible for zeaxanthin synthesis (Götz et. al., 2002), and plants with Norway spruce defence related phenol-oxidizing peroxidases (*spi 2*) showed marked tolerance to UV-B (Jansen et. al. 2001; Jansen et. al. 2004). Transgenic rice plants with increased levels of heat shock protein (sHSP17.7) exhibited significantly greater resistance to UV-B stress than untransformed control plants (Murakami et al 2004).

4. VACUOLE TRANSPORT ENGINEERING

The presence of large, acidic-inside, membrane bound vacuoles in many halophytic plants allows the efficient compartmentalization of sodium into the vacuole through the operation of vacuolar Na+/H+ antiports (Blumwald and Poole, 1985; Rausch et al., 1996; Apse et al 1999). Understanding of this stress adaptation – transport of sodium into the vacuole and ion homeostasis of plant cells - has tremendously increased in recent times (Martinoia et. al., 2000).

Interestingly transgenic tomato overexpressing this vacuolar antiporter gene accumulated sodium in its leaves but not in the fruits (Zhang and Blumwald, 2001). Transgenic *Arabidopsis* (Apse et. al., 1999), tomato (Drozdowicz et. al., 1999; Zhang and Blumwald, 2001; Zhang et. al., 2001), and *Brassica* (Zhang et. al., 2001) overexpressing a vacuolar Na+/H+ antiport gene from *Arabidopsis* (*AtNHX1*), were able to grow and produce in the presence of 200 mM sodium chloride without any reduction in yield or quality. The *A. thaliana* SOS 1 gene encodes a plasma membrane Na^+/H^+ antiporter that is essential for salt tolerance (Shi et. al., 2000, 2002, 2003; Qiu et. al., 2002). Similarly, many genes encoding proton pumps in cell membranes have successfully been modified since proton pumps generate the proton electrochemical gradients in the membranes. Gaxiola et. al. (2001) showed that transgenic plants overexpressing AVP1 H^+ pump were resistant to drought and salinity. Increased salt tolerance in the transgenic plants is correlated with reduced Na^+ accumulation under salt stress (Shi et. al., 2003). This work illustrates that it is possible to achieve dramatic improvements in plant stress tolerance by the manipulation of single target transporter gene, signal transduction pathways and gene regulation networks (Hasegawa et. al., 2000; Zhu, 2001).

5. REGULON ENGINEERING

Transcription factors have been identified as an additional target group to direct gene regulation for improved salt/drought stress tolerance (Shinozaki and Yamaguchi-Shinozaki, 1997; Winicov and Bastola, 1997; Broun, 2004). Understanding of stress signaling pathways has paved the way to this regulon engineering strategy. The

advantage of this technique is that only one regulatory gene needs to be overexpressed and the stress tolerance however, is due to the induction of many genes (Zhang et. al., 2004). There are three transcriptional activator CBF/DREB1 (C-repeat binding factor or dehydration responsive element binding) genes in Arabidopsis. Overexpression of a cDNA for cis-acting promoter element DREB1A in transgenic plants improved tolerance of plants to drought, salt loading, and freezing (Kasuga et al., 1999). When CBF1 was overexpressed in *Arabidopsis* it induced the expression of cold-regulated genes (COR) and increased freezing tolerance (Jaglo-Ottosen et. al., 1998; Stockinger et. al., 1997). Similarly, plants over-expressing *CBF3*, which shows constitutive accumulation of COR15am and COR6.6 proteins, and increased accumulation of proline and sucrose, develop greater freezing tolerance than wild-type after 7–14 d in the cold (Gilmour et al., 2000; Jaglo-Ottosen et. al., 1998; Kasuga et. al., 1999; Liu et. al., 1998). CBF4, a gene coding for a protein homologous to CBF/DREB1, when overexpressed in transgenic plants resulted in the expression of cold- and drought-induced genes under non-stress conditions, and improved the plants' tolerance to drought and freezing (Haake et. al., 2002). Transgenic *Arabidopsis* plants with high inducible levels of transcription factor AtMYB2, which is critical for the expression of *ADH* (alcohol dehyrogenase) gene showed higher drought resistance (Dolferus et. al., 2003). *Alfin1* cDNA from alfalfa encodes a novel member of the zinc-finger family of proteins and is modulated by sodium chloride. Transgenic overexpression of *Alfin1* in alfalfa enhanced the salinity tolerance (Winicov and Bastola, 1999). Overexpression of a zinc finger protein (SCOF-1) responsible for induction of COR gene expression resulted in enhanced low temperature tolerance in transgenic *Arabidopsis* and tobacco (Kim et al., 2002) Arabidopsis plants with transformed transcriptional activator gene (AB13) exhibited higher freezing tolerance due to enhancement of ABA-induced expression of genes for cold acclimation (Tamminen et. al., 2001). Vannini et al. (2004) demonstrated that transient expression of *Osmyb*4 gene from rice in *Arabidopsis* transactivates *PAL*2, *ScD*9 *SAD* and *COR*15*a* cold inducible promoters. *Myb4*-overexpressing plants showed a significant increase in cold and freezing tolerance. Overexpression of heat stress transcription factor (*HsfA1*) in tomato plants displayed higher thermotolerance (Mishra et. al., 2002). Following initial studies in *Arabidopsis*, many more investigations have demonstrated the validity of regulon engineering to achieve increased stress tolerance in several crops (Kim et. al., 2001; Park et. al., 2001; Hsieh et. al., 2002a and 2002b; Owens et. al., 2002; Dubouzet et. al., 2003; Shou et al., 2004).

6. CONCLUSIONS

Many stress tolerant mechanisms in plants have now been understood at least partially. Transgenic plants will continue to be used as important tools to dissect these mechanisms. As our understanding increases, further and new opportunities for metabolic engineering will emerge. We conclude that the following three avenues of re-

search are called for: 1. It should also be possible to improve plant stress tolerance by pyramiding several genes for different stress tolerance mechanisms in one transgenic crop. 2. Future studies are required to improve and evaluate crops engineered for stress tolerance for interacting stress factors; and 3. Studies are required to evaluate transgenic stress tolerance under field conditions.

The discovery of novel genes, determination of their expression patterns in response to abiotic stress, and an improved understanding of their roles in stress adaptation obtained by the use of "omics' technologies will provide the basis for metabolic engineering strategies leading to novel crop cultivars.

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CHAPTER 10

FUNCTIONAL GENOMICS OF STRESS TOLERANCE

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1. INTRODUCTION

A gene by gene approach has been normally used to understand function. Functional genomics allows large-scale gene function analysis with high throughput technology and incorporates interaction of gene products at cellular and organism level. The information coming from sequencing programs is providing enormous input about genes to be analyzed. The availability of *Arabidopsis* and rice genome sequence (The Arabidopsis Genome Initiative, 2000; Goff et al., 2002; Yu et al., 2002; Rice Genome Program, http://rgp.dna.affrc.go.jp/) has paved the way for studying the function of genes on a genome-wide scale. The non-availability of information about genomes from other plants will also be compensated in part by the availability of large collection of ESTs and cDNA sequences. The basic interest behind these EST projects is to identify genes responsible for critical functions. In many cases bioinformatics tools will come in handy. The gene function is defined by studying the transcripts, proteins and metabolites and also by altering the activity of a gene *per se*, e.g. by large-scale mutagenesis. These different ingredients of functional genomics have developed in their own right and termed as transcriptomics, proteomics, metabolomics, and phenomics (Chory et al., 2000; Holtorf et al., 2002). ESTs, cDNA libraries, microarray and serial analysis of gene expression (SAGE) are used to analyze global gene expression profiles in a functional genomics program. Complementing large-scale expression studies are large mutant collections. Though gene identification through physical and chemical mutagens has become amenable for large scale analysis with the availability of markers (Lukowitz et al., 2000), but gene tagging is more promising for functional analysis on a wider scale

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(Martienssen, 1998). This is because insertional mutagenesis not only causes a mutation but also tags that region and helps in its identification. The advantage of insertional mutagenesis is enhanced by the large collection of these mutants and their sequenced insertion sites available in public databases. Transgenics are commonly used for insertional mutagenesis. Analysis of function of a gene in one organism can lead to identify function of its orthologues in other organisms. These results however, can not be taken as absolute but only as preliminary clues for identifying gene function. For instance, the *Arabidopsis LEAFY* (*LFY)* controls the formation of floral meristems whereas the *LFY* homologue in rice, *RFL*, is involved in panicle branch initiation (Jeon and An, 2001).

One area of interest for functional genomics of plants is stress tolerance. Stresses reduce plant productivity, especially it has been reported that abiotic stresses account for the maximum loss of plant productivity compared to any other factor (Cushman et al., 1999). Efforts of plant scientists worldwide are to successfully bring their research to practical use in the field. The endeavor will be successful only when the understanding of the complexity of stress signaling and plant adaptive processes is complete. This would require analysis of the function of numerous genes involved in stress response by way of genomics approaches that would help assign function to each gene.

2. EXPRESSION GENOMICS

Gene expression is highly influenced and is up- or down-regulated by the environment. The expression of a gene or a group of genes during a specific stage reflects on functional relevance. Various methods are available for detecting and quantification of gene expression including northern blotting (Alwine et al., 1977), S1 nuclease protection (Berk and Sharp, 1977), EST library (Adams et al., 1991), differential display and its modifications (Liang and Pardee, 1992), Serial Analysis of Gene Expression or SAGE (Velculescu et al., 1995) and microarray (Duggan et al., 1999). Some of these providing information about dynamic state of expression, representing the whole genome, are discussed here.

2.1. ESTs and cDNA Library

Demarcation of a gene in eukaryotes is more difficult as compared to prokaryotes because their genes contain introns. Complementary DNAs (cDNAs) help in discovery of new genes, polymorphism and gene expression (Ewing et al., 1999; Mekhedov et al., 2000; Shannon et al., 2003; Zhu et al., 2003). The mRNAs which are found more abundant in tolerant genotypes or tissues under stress conditions might be expected to play an important role in stress tolerance and creating a cDNA library from these genotypes/ tissues would help in discovery of such genes and understanding their role in tolerance mechanisms.

Expression sequence tags (ESTs) represent nucleotide sequences of small portions of the expressed genes. These ESTs serve to tag and to fish out the putative genes and also help quantify their expression. ESTs are also found to be useful in understanding gene structure (Kan et al., 2001) and cloning of agronomically important genes using ESTs based physical maps (Kurata et al., 1997). The high throughput technology of sequencing in the last decade has helped in obtaining sequences of partial or complete cDNA clones and their subsequent identification through sequence homology with known ESTs or genes from other plant species or even other organisms. ESTs served as the central resource in studies of global gene expression through high-density microarrays and analysis of complex traits such as drought and salinity tolerance in *Arabidopsis* (Seki et al., 2001, 2002b), rice (Yamamoto and Sasaki, 1997; Kawasaki et al., 2001) and barley (Ozturk et al., 2002). In addition to *Arabidopsis* and rice, large scales EST projects are underway also for various crop species like maize (ZmDB, http://www.zmdb. iastate.edu/zmdb/EST), wheat (ITEC, http://wheat.pw.usda. gov/genome/) and sugarcane (SUCEST; http: sucest.lad.ic.unicamp.br) for general and specific traits. In the sugarcane EST project, 'SUCEST', more than 2,60,000 cDNA clones were partially sequenced from standard cDNA libraries generated from different tissues (Vettore et al., 2003). Analysis of SUCEST database identified 88 resistant gene analogs (Rossi et al., 2003) and 33 putative cold-regulated proteins (Nogueira et al., 2003) based on their homology using BLAST search. Nogueira et al. (2003) found several cold inducible genes, which have not been previously reported as being cold inducible, including those for cellulose synthase, ABI3-interacting protein 2, OsNAC6 protein and phosphate transporter by the analysis of around 1500 ESTs. Pih et al. (1997) identified 15 salt stress inducible genes with early, late or continuous patterns of expression in *Arabidopsis* after sequencing of 220 clones randomly from cDNA library. Bohnert et al. (2001) have constructed more than 50 cDNA libraries from plants, bacteria and fungi for comparative analysis of genes regulated under stress conditions.

In general, cDNA libraries represent a collection of transcripts and are likely to miss transcripts synthesized in lower amount. For rapid identification of differentially expressed genes, normalized or subtractive cDNA libraries (Bonaldo et al., 1996) provide a direct mean of enriching for unique cDNA species by eliminating common sequences. A number of highly effective methods are available to construct normalized and subtractive libraries (Soares et al., 1994; Bonaldo et al., 1996; Diatchenko et al., 1996; Kang et al., 1998; Carninci et al., 2000). In rice, a large number of candidate ESTs have been found related to abiotic stress by functional annotation and subtracted cDNA analysis (Babu et al., 2002; Sahi et al., 2003).

Functional annotation of full length cDNA in *Arabidopsis* (Seki et al., 2002a; Oono et al., 2003) and rice (Kikuchi et al., 2003) as well as rapid growth of EST databases, gene prediction and functional definition have sharply improved knowledge about transcriptome of plants. Its correlation with various stresses would help initiate investigation into plant stress responses.

2.2. Serial Analysis of Gene Expression

SAGE is a powerful technique that can be used for studying global gene expression profiles. This method relies upon the generation of unique short sequences, 9-17 base pairs (Velculescu et al., 1995; Saha et al., 2002) which contain sufficient information to identify a transcript (Fig. 1). This technique generates a large number of tags, can potentially identify >4^9 (>2,62,144) which is far more than the estimated number of genes in *Arabidopsis* (25,498, The Arabidopsis Genome Initiative, 2000) and rice (~45,000, Rice Genome Program, http://rgp.dna.affrc.go.jp/). The number of times a particular tag appears in a population of SAGE tags would provide direct information of cellular level of gene expression. This technique is much suited in those organisms whose genome has been sequenced. SAGE analysis has been extensively applied in yeast (Velculescu et al., 1997), humans (Zhang et al., 1997; Chen et al., 2002) and mouse (Gunnersen et al., 2002). Only a few studies have been conducted for analysis of global genes expression in plants by SAGE. Matsumura et al. (1999) and Gibbings et al. (2003) used SAGE for

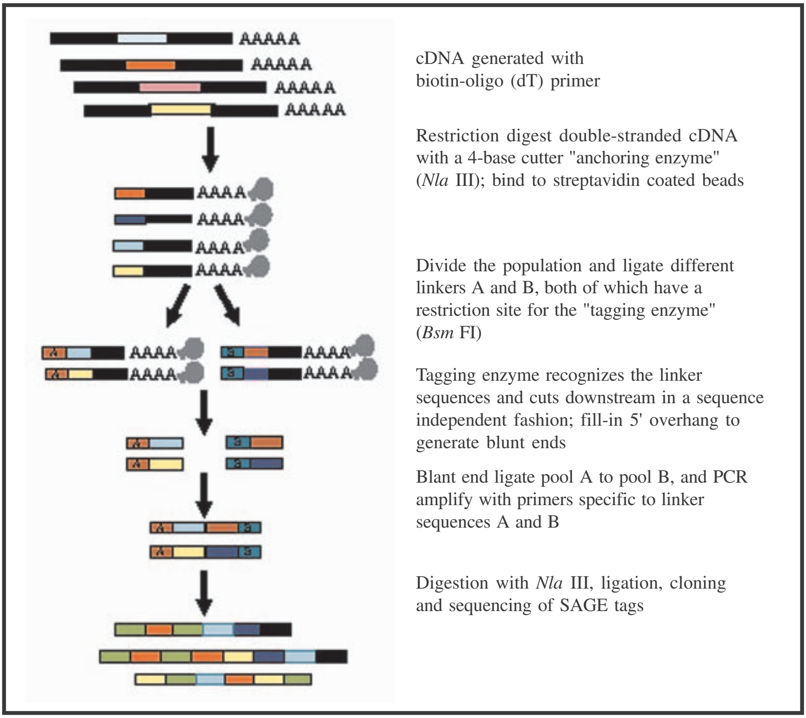

Figure 1. *Schematic diagram of SAGE (modified from Song, 2003)*

quantitative analysis of global gene expression in rice using 9-11 bp fragment tags. In rice seedlings, a total of 10,122 tags from 5921 expressed genes were analyzed and most of the tags belonged to the category of highly expressed housekeeping genes. Matsumura et al. (1999) also observed that among differentially expressed genes between anaerobically treated and untreated rice seedlings, metallothionein and globulin genes were highly expressed, and prolamin gene was most highly inducible. Eight genes, including six showing no match to any rice EST, were anaerobically induced and six genes were repressed. Global gene expression in rice leaf and seed revealed that out of 50,519 SAGE tags, 15,131 tags corresponded to unique transcripts and 70% occurred only once in both libraries (Gibbings et al., 2003). SAGE technology has also been used in soybean (Schupp et al., 2003) and tomato (Mysore et al., 2001) to analyze plant-pathogen interactions.

In *Arabidopsis*, SAGE technology was used to analyze changes in gene expression in leaves (Jung et al., 2003) and pollen (Lee and Lee, 2003) undergoing cold stress. A comparison of SAGE tags derived from cold-treated and normal leaves revealed that genes involved in cell rescue/defense/cell death/aging, protein synthesis, metabolism, transport facilitation and protein destination were highly expressed and photosynthesis genes were down-regulated. In case of pollen, twenty-six out of 21,237 were expressed at least 10-fold more in cold-treated compared to normal pollen. Out of highly expressed genes (more than 8-fold), only two genes were previously known to be involved in stress responses or defense but, there is no direct evidence that the function of these genes is related to cold stress.

By comparing the quantitative gene expression profiles of more than 10,000 tags between blast fungal (*Magnaporthe grisea*) elicitor- and buffer-treated control rice cells, 139 putative elicitor-induced genes and 154 repressed genes have been identified (Matsumura et al., 2003). Interestingly, 100 tags and 46 tags from elicitor-induced and -repressed genes could not match with the rice cDNA and EST data base reflecting on the need for enrichment of EST database. Among the down-regulated genes in the elicitor-treated cells, *Bl-1* gene coding for *Bax* inhibitor was identified. The expression of *Bl-1* is known to be involved in defense response in *Arabidopsis* (Sanchez et al., 2000) and barley (Huckelhoven et al., 2003).

2.3. Microarray

Microarray technology has revolutionized the analysis of global gene expression profiling. This technology is based on the principle of selective and differential hybridization of nucleic acids. Two types of microarray are mainly used, i.e. oligonucleotide based chip and cDNA microarray. DNA are spotted on a glass slide (DNA microarray) or on to a nylon membrane (DNA array) using the pin-based fluid transfer or inkjet dispensers (Fodor et al., 1991; Blanchard et al., 1996). Linker system to covalently immobilize modified nucleic acids (Beier and Hoheisel, 1999) or ATMS/diazotization technique (Dolan et al., 2001) can also be used to spot the DNA onto polypropylene

and glass slides. Microarrays are currently used for studying gene expression. The change in gene expression is measured by labeling the control and experimental transcript population with different fluorescent tags and then measuring the intensity and ratio of fluorescent signals bound to DNA microarray. This technology makes possible the rapid and comprehensive assessment of transcriptional activity during stress response and provides new insights into complex signaling networks governing these stress responses and helps identification of associated new genes. The plant microarray technology has been extensively reviewed recently (Baldwin et al., 1999; Schaffer et al., 2000; Aharoni and Vorst, 2002; Donson et al., 2002; Zhu, 2003b).

2.3.1. cDNA Microarray

The cDNA microarray is fabricated by robotically spotting PCR products resulting from direct amplification of genomic DNA by using EST-based or gene-specific primers on a glass slide (Jiao et al., 2003). A number of cDNA microarrays have been developed in plants, e.g. *Arabidopsis,* rice, maize, strawberry, petunia, ice plants and lima bean. Schena et al. (1995) first used the cDNA microarray to study the differential expression of 45 genes in roots and shoots in *Arabidopsis*. This high-throughput technology has been successfully used to analyze the regulation of genes at different stages of development (Lemieux et al., 1998; Kehoe et al., 1999) and in response to both abiotic and biotic stresses (Table 1). In an experiment to study salt stress, yeast transcripts analyzed using microarray showed that about 300 transcripts (~5% of all open reading frames) have at least two-fold increase in abundance and about 200 genes were down-regulated to a similar extent (Cushman and Bohnert, 2000). This study is expected to provide identification of genes and functional information of cellular tolerance mechanisms that are evolutionarily conserved. Seki et al. (2002b) prepared a 7,000 full length cDNA microarray in *Arabidopsis* to identify genes related to drought, cold, or salinity and to examine the differences and cross-talk between their signaling cascades. In total, 277 drought-inducible, 53 cold-inducible and 194 salinity-inducible genes were identified. Only 22 genes were identified as drought-, cold- and salinity-inducible genes, while 70% of salinity-inducible genes were also induced by drought stress, which indicated a strong correlation between drought and salinity stress response. In maize, the early post-pollination phase development is particularly sensitive to water deficit (Yu and Setter, 2003). Using cDNA microarray, it was found that in placenta, the major class of genes which was up-regulated included stress tolerant proteins like heat shock proteins, chaperonins, and major intrinsic proteins.

Maleck et al. (2000) have applied microarray technology using *Arabidopsis* microarray containing 10,000 expressed sequence tags (~7000 genes), representing 25-30% of total *Arabidopsis* genes, to analyze transcriptional programming during the systemic acquired resistance (SAR) under 14 different chemical and biological conditions related to SAR. The expression of 413 ESTs was differrent during 14 SAR experiments. In another experiment, transcriptional changes of 2,375 genes have been ana-

lyzed after inoculation with the fungal pathogen *Alternaria brassicicola* and defense-related signaling molecules, i.e. salicylic acid, methyl jasmonate or ethylene, using the *Arabidopsis* DNA microarrays (Schenk et al., 2000). It was found that 705 ESTs showed differential expression in one or more of these experiments. A microarray enriched in wound and insect responsive sequences has been used to analyze expression in *Nicotiana longiflora* in response to solar ultraviolet-B radiation and *Manduca sexta* herbivory elicitor (Izaguirre et al., 2003). Many photosynthesis-related genes were down-regulated and genes involved in fatty acid metabolism were up-regulated. Interestingly, UV-B and insect herbivory treatment similarly regulate the expression of genes encoding a WRKY transcription factor and several other insect-responsive genes of unknown function.

2.3.2. Oligonucleotide Microarray

Fodor et al. (1991) utilized solid-phase chemistry, photolabile protecting groups and photolithography to chemically synthesize oligonuleotides directly onto solid substrate for *in-situ* fabrication of arrays. The design of oligonucleotide sequences is based on the information available in databases and thus there is no need to maintain a large collection of cloned DNA molecules. To understand the response to wounding and interaction between wounding, pathogen, abiotic stress and hormonal response, *Arabidopsis* Genome GeneChip arrays (Affymetrix, Santa Clara, CA) were used (Cheong et al., 2002). Many osmotic stress and heat shock regulated genes were highly responsive to wounding although a number of genes involved in ethylene, jasmonic acid and abscisic acid pathways were also activated in response to wounding. A 20-mer maize GeneChip has also been designed from 1,500 ESTs to study differentially expressed genes in leaf tissues infected with fungal pathogen (Simmons et al., 2002). For increasing the specificity, 50-mer and 70-mer probe arrays have also been designed and it was found that there were no difference between these arrays and full length cDNA arrays (Kane et al., 2000; Ten-Bosch et al., 2001). A 25-mer oligonucleotide array (GeneChip Rice Genome Array) representing 21,000 genes of rice cultivar Nipponbare was used to study the gene expression during different stages of rice grain filling and to identify genes involved in synthesis and transport of carbohydrates, proteins and fatty acids (Zhu et al., 2003). Using the same GeneChip, Cooper et al. (2003) analyzed the expression profile during stress response and seed development. They have found that several genes which respond to environmental cues and stresses may also have a role in development.

3. MUTANTS

A direct way of characterizing gene function is to mutate the gene and study the consequence. However, effective means are not available for targeted gene replace-

ment in flowering plants. Gene expression can also be repressed by use of antisense, co-supression or RNAi strategies, but these approaches are largely based on a single gene approach and presently are difficult to use on a large scale (Parinov and Sundaresan, 2000). Physical and chemical means of mutagenesis have largely given way to insertional mutagenesis for the benefit of genome-wide studies.

Table 1. *Use of microarrays for analyzing transcriptional profile under stress conditions*

Plants	*Traits studied*	*Microarray*	*Reference*
Arabidopsis thaliana	SAR response	a-10000	Maleck et al. (2000)
Arabidopsis thaliana	Mechanical wounding and insect feeding	a-150	Reymond et al. (2000)
Arabidopsis thaliana	Defense-related signaling molecules and fungal pathogen	a-2375	Schenk et al. (2000)
Phaseolus lunatus	Herbivory (spider mite infestation) or herbivore-induced volatile substance	a-2032	Arimura et al. (2000)
Arabidopsis thaliana and *Cryophytum crystalinum*	High salinity	a-9212 *A. thaliana* and a-2600 *C. cyrstalinum*	Bohnert et al. (2001)
Arabidopsis thaliana	Oxidative stress	a-11000	Desikan et al. (2001)
Arabidopsis thaliana	Stress-induced and -repressed genes	a-1200	Mahalingam and Fedoroff (2001)
Arabidopsis thaliana	Drought and cold stress	a-~1300	Seki et al. (2001)
Arabidopsis thaliana	Iron deficiency stress in roots and shoots	a-6000	Thimm et al. (2001)
Oryza sativa	Initial phase of salt stress	a-1728	Kawasaki et al. (2001)
Arabidopsis thaliana	Water-deficit stress	a-1300	Bray (2002)
Arabidopsis thaliana	Wounding	b-~8200	Cheong et al. (2002)

Table 1. *Continued...*

Arabidopsis thaliana	Low-oxygen response	a-3500	Klok et al. (2002)
Arabidopsis thaliana	Salt, osmotic and cold stress	b-~8100	Kreps et al. (2002)
Arabidopsis thaliana	L-proline treatment	a-7000	Satoh et al. (2002)
Arabidopsis thaliana	Infection with *Pseudomonas syringae* pv. *tomato*	a-13000	Scheideler et al. (2002)
Arabidopsis thaliana	Drought, cold and high-salinity stress	a-7000	Seki et al. (2002b)
Hordeum vulgare	Drought and salinity	a-1463	Ozturk et al. (2002)
Lycopersicon esculentum	Response to the fungal toxin fusicoccin (FC)	a-235	Frick and Schaller (2002)
Oryza sativa	Stress, especially UV-B and gamma irradiation	a-1265 (phase I), 8978 (phase II)	Kikuchi et al. (2002)
Arabidopsis thaliana	Pollen under cold stress	a-712	Lee and Lee (2003)
Arabidopsis thaliana	High light stress	a-7000	Kimura et al. (2003)
Arabidopsis thaliana	Inoculation with *Alternaria brassicicola* and treatment with signal molecules, reactive oxygen species-inducing compounds and UV-C	a-~7000	Narusaka et al. (2003)
Arabidopsis thaliana	Cation stress	b-1096 (2003)	Maathuis et al.
Arabidopsis thaliana	Rehydration process after dehydration	a-7000	Oono et al. (2003)
Arabidopsis thaliana	Vitamin C-deficient mutant	b-8200 (2003)	Pastori et al.
Arabidopsis thaliana	Response to *Pseudomonas syringae*	b-~8000	Tao et al. (2003)

Table 1. *Continued...*

Arabidopsis thaliana	Responses elicited by diverse RNA viruses in susceptible hosts	b-~8300	Whitham et al. (2003)
Oryza sativa	Environmental, biological and chemical stress treatments	b- 21,000	Cooper et al. (2003)
Zea mays	Endosperm and placenta/ pedicel tissues in developing kernels under water stress	a->5000	Yu and Setter (2003)
Nicotiana tabacum	Resistance against oomycetes	a- 298	Hugot et al. (2004)
Populus euphratica	Salt stress	a- 315	Gu et al. (2004)

a: cDNA microarray; b: oligonucleotide microarray

3.1. Positional Cloning

The traditional approach to generate mutants through chemical or physical mutagenesis and identify mutants by using screens for specific stresses has been found useful. Access to the mutated gene is obtained using various positional cloning strategies. The map-based cloning strategy identifies the gene based on mutant phenotype by identifying linkage to markers whose physical location in genome is already known. The disadvantage of this method of identifying genes for stress response was the effort required to make a physical map to develop markers for identifying the genetic locus to be followed by cloning, determining the sequence of the region and validation of gene function through complementation (Jander et al., 2002). Thus, such a method could not be used for large-scale gene identification. There have been several advances like the sequencing of the *Arabidopsis* and rice genomes, availability of markers and advances in methods to detect DNA polymorphisms including SNPs, over the past few years due to which positional cloning can be considered as an option for functional genomics. The Arabidopsis Information Resource (TAIR) provides integrated data for over 4,000 genetic markers and 90,000 polymorphisms (Garcia-Hernandez et al., 2002). Similarly, a large number of markers are also available for rice, including 3267 markers available at RGP, Japan (http://rgp.dna.affrc.go.jp/publicdata/genetic map 2000/ index.html), 171 CAPS markers (Harushima et al., 1998) and over 2000 SSR markers (Akagi et al., 1996; Chen et al., 1997; Temnykh et al., 2000; McCouch et al., 2002).

Positional cloning has helped identify several genes in the stress signaling pathway. These include *SOS1*, a Na^+/H^+ antiporter (Shi et al., 2000), *SOS2,* a Ser/Thr protein kinase (Liu et al., 2000), *SOS3*, a calcium binding protein (Liu and Zhu, 1998), *SOS4*, a pyridoxal kinase (Shi et al., 2002), *SOS5,* a cell surface adhesion protein (Shi et al., *2003*), *HOS1*, a ring finger protein which negatively regulates low-temperature-responsive gene transcription (Ishitani et al., 1998; Lee et al., 2001), *SpI7*, a heat stress transcription factor (Yamanouchi et al., 2002), *STT3*, subunit of oligosaccharyltransferase complex which controls adaptive response to salt/osmotic stress (Koiwa et al., 2003), *FRO1*, a NADH dehydrogenase subunit of mitochondrial respiratory chain complex I which is involved in regulation of nuclear gene expression in response to cold (Lee et al., 2002) and *LOS5/ABA3*, a molybdenum cofactor sulfurase that modulates cold and osmotic stress responsive gene expression (Xiong et al., 2001). Several genes involved in biotic stress signaling have also been identified through this approach; these include *Xa21*, which confers resistance to bacterial pathogen *Xanthomonas Oryzae* (Song et al., 1995), *Pib*, which confers rice blast resistance (Wang et al., 1999), *Pita2* (Bryan et al., 2000), *Pi5(t)* (Jeon et al., 2003), *Xa1* (Yoshimura et al., 1996) and *Xa26* (Yang et al., 2003).

Several mutagens produce sizable deletions like diepoxybutane, fast neutron and gamma rays. A collection of 40,000 deletion mutants has been made in rice in the IR64 background using these mutagens (Leung et al., 2001). Analysis of 10,000 of these mutant lines for alteration in stress signaling revealed at least 0.3% of the mutants to be altered in the disease response pathway. Abiotic stress tolerance was also assessed in the mutant populations. A total of 3,000 lines were studied for submergence stress, of which six mutants were identified which showed ~75% recovery after submergence stress. Under drought conditions, 26 out of 1200 plants showed better response compared to wild type. These mutants have only been studied at the physiological level but the molecular basis of these alterations in stress response is still to be determined.

Apart from deletion mutants, a high throughput technique known as TILLING (Targeting Induced Local Lesions In Genomes), for reverse genetics is available which can detect single base changes. The approach makes use of high-density point mutations generated using chemical mutagenesis. These populations are screened for mutations using denaturing high performance liquid chromatography (DHPLC) (McCallum et al., 2000) or endonuclease, which would specifically cleave mismatches in a heteroduplex between wild type and mutant (Colbert et al., 2001). Users can make use of the TILLING facility through an interactive web based system; http://www.proweb.org/coddle and the mutant collection consisting of about 10,000 lines can be accessed through Arabidopsis Biological Research Center (ABRC, Ohio State University). TILLING has been recently used to discover polymorphism in natural populations. This approach called Ecotilling, was used to study variation in 192 accessions of *Arabidopsis*. Study of different ecotypes through TILLING may help identify gene function, e.g. a null allele was identified by Ecotilling amongst the different accessions for *DRM1* (*DOMAINS REARRANGED DNAMETHYLASE1*) gene showing that the gene is dispensable (Comai et al., 2004).

3.2. Insertional Mutagenesis

Insertion of a known segment of DNA into a gene of interest is a commonly used strategy for mutagenesis. Insertional mutagenesis offers a more rapid mean to clone a gene as the insertion not only creates a mutation but also serves to tag the region, which helps in its identification (Bouchez and Hofte, 1998). The two most commonly used tools for insertional mutagenesis in plants are use of transposons or T-DNA tags (Krysan et al., 1999). T-DNA is a segment of the tumour-inducing (Ti) plasmid of *Agrobacterium tumefaciens* and is delimited by short imperfect repeat border sequences. The T-DNA including any region between its left and right border can be transferred by *Agrobacterium* into the plants where the T-DNA gets inserted in the plant genome. The mutants are screened based on unique sequence within the left and right border, for instance a selectable marker gene or a reporter gene (Azpiroz-Leehan and Feldmann, 1997). T-DNA tagged lines have been generated in *Arabidopsis* with the specific aim of isolating mutants altered in stress signaling. These tagged lines have been generated in the transgenic background of a stress inducible *RD29A* promoter (responsive to cold, salt, dehydration and ABA) fused to *luciferase* reporter gene. Luciferase enzyme activity was monitored in response to several stresses. On this basis, three groups of mutants have been identified, *cos* (constitutive expression of osmotically responsive genes), *los* (low expression of osmotically responsive genes) and *hos* (high expression of osmotically responsive genes) (Ishitani et al., 1997). Preliminary screening of this collection identified 43 mutants in the '*hos*' category and 20 in the '*los*' category (Bohnert et al., 2001). Several important genes in abiotic stress pathway have been identified using these mutants (Table 2). Jeon et al. (2000) developed 22,090 T-DNA tagged lines in rice of which 18,358 were fertile. These lines have recently been used for identifying low-temperature-responsive genes in rice (Table 2). Lee et al. (2004) analyzed 15,586 rice lines, out of which 81 (0.52%) showed cold-responsive GUS expression. Also, 37 tagged genes were identified from these lines, two of which, *OsRLK1* (putatitive LRR-type-receptor like protein kinase) and *OsDMKT1* (putative demethylmenaquinone methyl transferase) were confirmed experimentally to be cold-responsive.

Transposable elements have also been used on a genome-wide scale for functional analysis. Endogenous transposable elements have been identified and characterized in several plant species (Ramachandran and Sundaresan, 2001). Transposons have been used in heterologous systems also to identify gene function, *Activator*/*Dissociation* (*Ac*/*Ds*) and *Enhancer*/*Supressor-mutator* (*En*/*Spm*) being two most widely used transposable elements for insertional mutagenesis (Parinov and Sundaresan, 2000). The maize *En*/*Spm* element was used for generating 48,000 *Arabidopsis* lines. A total of 420 tagged genes were identified based on analysis of 1,200 flanking sequences (Tissier et al., 1999). Using *En/Spm* elements, Speulman et al. (1999) reported the production of 2,592 lines. Around 250 flanking sequences were analyzed from which 100 tagged genes were identified but none of them were found to be involved in stress response. For characterizing the MYB family of genes, three transposon tag collections (Baumann et al., 1998; Speulman et al., 1999; Tissier et al.,

Table 2. *List of some genes in the stress signaling pathway identified through insertional mutagenesis by T-DNA*

Gene	*Stress*	*Function*	*Reference*
AtHKT1 (*Arabidopsis thaliana* high affinity K^+ tranporter)	Salt	Controls sodium entry into plant roots	Rus et al. (2001)
**AtCPL1* (*Arabidopsis thaliana* C-terminal domain phosphatase-like)	Cold, ABA, salt	Negative regulator of stress gene transcription	Koiwa et al. (2002)
**AtCPL3* (*Arabidopsis thaliana* C-terminal domain phosphatase-like)	ABA	Negative regulator of stress gene transcription	Koiwa et al. (2002)
**OSM1/SYP61* (*Oryza sativa* syntaxin)	Salt, drought, ABA	Stomatal response to ABA	Zhu et al. (2002)
CBL1 (*Arabidopsis thaliana* calcineurin B-like protein)	Cold, salt, drought	Positive regulator of drought and salt and negative regulator of cold stress	Cheong et al. (2003)
CIPK3 (*Arabidopsis thaliana* calcium associated protein kinase)	ABA, cold, salt	Cross talk between cold and ABA signal transduction	Kim et al. (2003)
OsRLK1 (*Oryza sativa* putative LRR-type receptor-like–protein kinase)	cold	Cold response	Lee et al. (2004)
OsDMKT (*Oryza sativa* putative demethyl menaquinone methyl transferase)	cold	Cold response	Lee et al. (2004)

** Mutants generated in RD29A:LUC background*

1999) and one T-DNA tagged collection (Bouchez et al., 1993) were used. A total of 47 insertions were identified in 36 members of the R2R3 MYB family. Screening for an altered phenotype in 32 lines representing disruptions in 26 MYB genes showed no distinct phenotype in most of the lines analyzed using screens for stress among others with the exception of a UVB stress tolerant line (Meissner et al., 1999).

A modified approach to the use of T-DNA and transposon tagging for insertional mutagenesis is the use of enhancer elements in the construct. These enhancers

may cause transcriptional activation of nearby genes, hence this approach is referred to as activation tagging. Weigel et al. (2000) reported a large collection of such mutants in *Arabidopsis* using a T-DNA vector with four copies of CaMV 35S enhancer element. Several different stresses were used as screens to study these mutants. In the screen for disease resistance, two mutants were characterized at the molecular level, one of them showing a disease resistant (*cdr1-1D*) and the other showing a disease sensitive (*cds1-1D*) phenotype were identified.

Mutant studies provide valuable data for functional validation of gene function, but since this is also a transgenic approach, environmental issues are naturally raised (Hirochika, 2001). To overcome these concerns, endogenous transposons are desirable. *Tos17*, a rice retrotransposon, has been used for large-scale mutagenesis (Miyao et al., 2003). *Tos17* is activated only in tissue culture conditions and is not active under normal conditions. There are only two copies of *Tos17* under normal conditions in japonica rice and 5-30 transposed copies are found in plants regenerated from tissue culture. A total of 47,196 lines were generated through tissue culture. Analysis of flanking sequences from 4,316 lines showed that disease/defense related genes constituted a total of 13.8% of the total of 16,784 independent sequences analyzed.

A number of genes have been identified through insertional mutagenesis whose functional analysis is underway (Table 2). There are largely two strategies for screening insertion lines generated through T-DNA and transposon tagging. One is a 'pooling' strategy in which ~20-100 insertion lines form a pool and these pools can be combined to form super pools so that PCR screening can be done in stages. PCR screening of these pools is done using gene- and insert-specific primers. An alternative to this strategy is sequencing of regions flanking the insertion-site. Though this strategy is definitely more time consuming, but it provides more precise information so that one can easily search for a particular gene of interest in the database by BLASTN searches. These flanking sequence tag (FST) databases can be made more useful by linking them to phenotype information (Hirochika et al., 2004). These databases for sequenced flanking insertion sites will expand in the near future and make the task of searching the databases available for reverse genetics much easier as this will lead to shift from chance-driven to sequence-driven screens for mutant analysis (Krysan et al., 1999).

3.3. Traps

A significant drawback in the use of mutants for functional analysis is that the phenotype is not always evident on mutating the gene which can happen because of functional redundancy of the genes or because the mutation is conditional and there is no evident morphology even in the presence of severe physiological defects (Springer, 2000; Bouche and Bouchez, 2001). To overcome such problems and complement other efforts, traps: modified tags containing a reporter gene, have been developed. There are three basic kind of traps, gene, enhancer and promoter traps. The expression pattern of

trapped reporter gene gives clues about possible function while the gene itself serves as tag. Enhancer traps contain a reporter gene fused to a minimal promoter. The reporter gene will be expressed only when enhancer elements are close to the site of integration. Promoter traps contain a promoter-less reporter gene and expression will be seen only when the insertion is in an exon and that too in the correct orientation. On the other hand, a gene trap contains a reporter with one or more splice acceptor sites preceding it. In this case, expression will occur only if insertion occurs in an intron (Springer, 2000). The bacterial *gusA* gene and the jellyfish green fluorescent protein gene (*GFP*) are the two most commonly used reporter genes for traps. A modified tag intended to be used as a promoter trap was used for the first time by Koncz et al. (1989). A T-DNA vector was used with a promoter-less *aminoglycoside phosphotransferase* (*aphII*) as a reporter gene. Sundaresan et al. (1995) reported the use of gene and enhancer traps using the *Ac/Ds* family of maize transposons in *Arabidopsis* using GUS as a reporter. GUS expression could be detected in about 50% of enhancer trap lines and 25% of gene trap lines. Several large collections of traps are now available for functional analysis (see section on resources). In rice, a total of 31,443 enhancer trap lines were generated using GAL4/VP16-UAS elements and GUS as a reporter (Wu et al., 2003). A collection of 20,261 transgenic *Arabidopsis* lines were generated using a promoterless firefly *luciferase* reporter gene. 3.7% of these mutants showed altered luciferase expression on exposure to stress. Detailed analysis of one of these lines showing salt inducible luciferase expression helped identify an ABC transporter, *At1g17840*, the expression of which is regulated by sugar, salt, ABA and GA (Alvarado et al., 2004). It may, however, be mentioned that an expression in these mutants need not reveal involvement of gene in stress tolerance and further validation of gene function is required.

4. TRANSGENICS

There are several reports where transgenic systems have been used to study gene expression involved in stress response (Tyagi and Mohanty, 2000; Xiong et al., 2002; Zhu 2002, 2003a). The function can be studied either by overexpression (Cheong et al., 2003; Mukhopadhyay et al., 2004) or suppression of gene expression using antisense or RNAi strategy (Koiwa et al., 2003; Xiong and Yang, 2003). Transgenics have also been used to study stress responsive promoters (Haralampidis et al., 2002; Trinidade et al., 2003). But the major limitation of this gene-by-gene approach is that it is still not amenable for large-scale studies. There are very few reports where a transgenic approach has been used for large-scale functional analysis. One such large-scale approach of using trasngenics for functional analysis involves the use of plant artificial chromosome (PLAC) containing genome fragments having a large number of genes. This would greatly reduce the number of transgenics required for analysis. If we consider the *Arabidopsis* genome, only around 500 plants would be required provided the PLACs have on an average of 50 genes (Somerville and Somerville, 1999).

The first large-insert library for raising transgenics was made for *Arabidopsis*. The library was made in a BIBAC2, a plant-transformation-competent binary vector. The library represents 11.5X coverage of the *Arabidopsis* genome and would greatly aid gene identification through complementation screening (Chang et al., 2003). A large-scale gene inactivation through transgenic approach can be achieved by RNA mediated gene silencing. As part of the European Consortia's effort for *Arabidospis* functional genomics, the AGRIKOLA (*Arabidopsis* Genomic RNAi Knock-Out Line Analysis) has been set to make a collection of ~ 50,000 binary vectors for gene silencing using RNAi for every *Arabidopsis* gene. The target will be to transform *Arabidopsis* with around 5000 of these plasmids with the aim of targeted gene silencing; details of these vectors can be obtained through www.agrikola.org (Hilson et al., 2003).

5. PROTEOMICS

The large-scale functional analysis of gene products, being expressed as proteins, is called proteomics (Pandey and Mann, 2000). It involves identification, quantification, activity and molecular interactions of all proteins from an organism. A proteome is a total protein complement of a genome (Wasinger et al., 1995) and the analysis of a functional proteome provides a powerful tool for linking gene expression to cell metabolism and also to genetic maps (Burstin et al., 1993). Proteomics tells us what fraction of the genome is functional and at what levels. Proteome analysis provides better annotation of genome sequences by taking into account very small open reading frames (ORFs) which are functional. It can also assign an unidentified ORF to a particular protein product. Protein micro-characterization and their post-translational modifications can be determined by proteome methodologies (Park, 2004).

Proteomics concept is based upon high quality separation of proteins. This science of proteins dates back to late 1970's with the advent of two-dimensional gel electrophoresis (2DGE) (O'Farrell, 1975). The proteins are separated by 2DGE, estimated using image analysis and then the mass spectrometer based identification of gel separated proteins is carried out. Two-dimensional electrophoresis separates peptides according to their molecular mass and isoelectric point. The 2DGE is the most powerful technology in proteomics for separating a few thousands proteins simultaneously. Proteomics approach is dependent on high purity and reproducibility. This approach has been used to identify wound-response related proteins in rice (Shen et al., 2003).

Several computer softwares have been developed for the quantitative analysis of complex 2D patterns but the major advancement in proteomics has been a combination of 2D with mass spectrometry (MS) in 1990's (Yates, 1998). MS technique has replaced the sensitive methods of Edman's protein microsequencing which has remained time and cost intensive. MS is a high throughput technique making possible the analysis of hundreds of proteins in short time and with picomole amounts of protein (Burlingame et al., 1996; Blackstock and Mann, 2001; Mann et al., 2001; Mann and Pandey, 2001). The basic principle is to generate ions from a source and analyze these

ions according to their masses. MALDI-TOF (matrix-assisted laser desorption/ionization-time of flight) is one such source that generates peptide fingerprints resulting from digestion of protein with proteases, trypsin being the most commonly used protease. The mass spectrum of eluted peptide mixture results in 'peptide mass fingerprints' which can be compared with the predicted sequences from genomes or cDNA available in databases (Henzel et al., 1993). MALDI-TOF identification procedure may not be appropriate in cases where full-length genes are not sequenced. Another source, electro spray ionization (ESI) provides more information via fragmentation of individual peptides (Pandey and Mann, 2000). The peptide ions are fragmented by collision to isolate one species at a time producing different amino and carboxy terminal fragments. Peptide masses are compared to available databases and analyzed. This gives more specific information than MALDI fingerprinting. It not only provides information for protein sequence databases but also for nucleotide sequences such as EST databases (Kuster et al., 2001). In *Arabidopsis*, nuclear proteome has been comprehensively characterized in response to cold stress using 2D gel electrophoresis and MALDI-TOF-MS (Bae et al., 2003). Out of 184 protein spots, 40 proteins were induced and 14 repressed in response to cold stress by more than two folds. Many of them were already known to be involved in stress, including heat-shock proteins, transcription factors (AtMYB2 and OBF4), DNA binding proteins (DRT102 and Dr1), catalytic enzymes, syntaxin, calmodulin, and germin-like proteins. Similar study of proteome analysis in wheat grains following heat stress identified a total of 37 proteins which were significantly changed by heat treatments (Majoul et al., 2003). Costa et al. (1998) in a proteomic study of drought stress in maritime pine quantified about 1000 proteins and found 38 were stress responsive. Similarly, in rice, out of >1000 quantified proteins from stressed and well watered rice leaves, 42 proteins were found responsive to stress (Salekdeh et al., 2002). Eight proteins showing reversible behavior in leaves under drought stress were identified by tryptic fingerprinting with MALDI-TOF MS and partially sequenced with ESI-Q-TOF MS/MS. There are several examples where proteins associated with various processes affecting response to stress conditions have been identified, e.g. maize (Riccardi et al., 1998) and wild watermelon (Kawasaki et al., 2000).

Protein chips are particularly useful for assessing and analyzing protein diversity on a large scale including hydrophobic and large proteins that are difficult to visualize by 2D method. This approach is based on use of arrays for proteome analysis. The surface of protein chips can be immobilized with antibodies or recombinant proteins into an array format (Lueking et al., 1999). The protein chip is probed with a sample of interest. Different fluorescently labeled cell lysates can be added and proteins bound to chip surface are detected by a change in colour. The bound proteins can be eluted and identified by mass spectrometry. Alternatively, instead of cell lysates, phage cDNA display library can be added to the chip. Phage display method expresses the protein of interest fused to coat protein of bacteriophage (Li, 2000). Immobilized proteins of the chip can capture proteins displayed on phage particles. The cDNA of interest expressing the desired protein can be sequenced to know about the interacting proteins. In

yeast, a proteome chip has been constructed using 5800 proteins and screened for their ability to interact with proteins and phospholipids (Zhu et al., 2001).

Yeast two hybrid system can also be used for determining protein-protein interactions (Field and Song, 1989). Yeast clones containing ORFs are fused to DNA binding or activation domains, whereby proteins encoded by ORFs interact in the cell nucleus. ORF clones are then identified by sequencing. Large-scale analysis with array is also carried out. Such arrays are probed with single ORF DNA binding domain fusions. The surviving colonies on the basis of nutritional selection identifies the interacting proteins which can be sequenced directly. The hybrid system can be automated and provides a high throughput technique for studying protein interactions (Walhout and Vidal, 2001).

Proteomics-based research is proving helpful in characterization of stress-related proteins and identification of corresponding novel stress-responsive genes for production of stress tolerant plants.

6. RESOURCE FOR FUNCTIONAL GENOMICS

The efforts to understand gene function by individual groups can never gain the required momentum for large-scale functional genomics till there is a true sharing of resources amongst the scientific community. This would be made possible by putting the resources into easy-to-use, well-catalogued and freely accessible databases to the research community. A large number of databases are now available for public use containing details of mutant collections and EST/cDNA sequences to facilitate the task of functional analysis (Tables 3 and 4).

The *Arabidopsis* Information Resource (TAIR) is the most well integrated platform for *Arabidopsis* researcher's world wide including data contributed by over 11,000 researchers and 4,000 organizations available at http://arabidopsis.org (Rhee, 2000; Rhee et al., 2003). This site not only provides the details of *Arabidopsis* genome sequence but also information regarding annotation, gene families, maps, markers, polymorphisms, protein information, metabolic pathways and gene expression data as well as seed and DNA stocks. The data available at TAIR includes around 400,000 nucleotide sequences, 200,000 mutant lines, 400 genetic markers, 90,000 polymorphisms and data from more than 600 microarray experiments (Garcia-Hernandez et al., 2002). Keeping pace with the completion of the rice genome sequence, several databases are available for rice functional genomics too. These include the Rice Genome Program (RGP, Japan; http://rgp.dna.affrc.go.jp/); Arizona Genomics Institute (AGI; http://www.genome.arizona.edu/); The Institute of Genomic Research Rice Database (TIGR; www.tigr.org/tdb/rice), International Rice Information System (IRIS; www.iris.irri.org) and Oryzabase (http://www.shigen.nig.ac.jp/rice/ oryzabase/top.jsp). The Rice Genome Research Center (RGRC), established by the National Institute of Agrobiological Sciences, Japan in 2003 is accessible through RGP's site. This site provides access to ~50,000 *Tos17* insertion lines of rice and flanking sequences of 5,000 lines. It also

Table 3. *Some Resources for functional genomics*

Source	Resource	Website	Reference
Institute of Molecular Agrobiology, Singapore	931 *Ds* insertion lines *(Arabidopsis)*	http://www.plantcell.org/cgi/content/full/11/12/2263/DC1	Parinov et al. (1999).
Nottingham *Arabidopsis* Stock Center (NASC), England	T-DNA lines, Enhancer trap lines, Activation tagged lines *(Arabidopsis)*	http://nasc.nott.ac.uk/ima.html	Wilson et al. (1999).
The *Arabidopsis* Knockout Facility (University of Wisconsin), USA	60,480 T-DNA tagged lines, 72,960 Activation tagged T-DNA lines *(Arabidopsis)*	http://www.biotech.wise.edu/Arabidopsis	Sussman et al. (2000).
Syngenta *Arabidopsis* Insertion Library (SAIL), USA	52,964 T-DNA tagged lines *(Arabidopsis)*	www.tmri.org	Sessions et al. (2002).
MaizeGDB: Maize Genetics & Genomics Database, USA	361,014 ESTs (maize)	http://zmdb.iastate.edu	Dong et al. (2003).
Maize-targeted Mutagenesis database (mtmDB), Cold Spring Harbor Laboratory, USA	43,776 Mutator lines (maize)	http://mtm.cshl.org/flow.html	May et al. (2003).
Pohang University of Science and Technology (POSTECH), Korea	22,090 T-DNA tagged lines (Rice)	genean@postech.ac.kr	Jeon et al. (2000).
Knowledge-based *Oryza* Molecular Biological Encyclopedia Database (KOME), Japan	~30,000 cDNA clones (rice)	http://cdna01.dna.affrc.go.jp/cDNA	Kikuchi et al. (2003).

Continued...

Table 3. *Continued...*

Source	**Resource**	**Website**	**Reference**
National Institute of Agrobiological Sciences, Japan	47,196 *Tos 17* induced insertion mutants (rice)	http://tos.nias.affrc.go.jp/miyao/pub/tos17/	Miyao et al. (2003).
Rice Mutant Database, Huazhong Agricultural University, China	~31,443 Enhancer trap T-DNA lines (Rice)	http://www.ricefgchina.org/mutant/	Wu et al. (2003).
Zhejiang University China	1000 T-DNA tagged lines (Rice)	http://www.genomics.zju.edu.cn/ricetdna	Wu et al. (2003).
Tomato digital expression database, USA	1,50,000 ESTs (tomato)	http://www.tigr.org/tdb/lgi	Van der Hoeven et al (2002).
Sugarcane EST database, Brazil	2,60,000 ESTs (Sugarcane)	http://sucest.lad.dcc.unicamp.br/en/	Vettore et al. (2003).

provides access to a total of ~30,000 full length cDNA clones. More information regarding these clones can be searched from the Knowledge-based Oryza Molecular Biological Encyclopedia database (KOME; http://cdna01.dna.affrc.go.jp/cDNA). AGI provides BAC and EST libraries, high density hybridization filters and clones on a cost recovery basis. Databases dedicated to comparative cereal genomics include USDA Gramene database (http:// www.gramene.org; Ware et al., 2002a, b) and John Innes Cereal Databases (www.jic.bsrc.ac.uk). TIGR provides details of both rice and *Arabidopsis* genome sequencing, the site also provides details of 13, 659 *Tos17* insertion flanking sequences mapped *in silico* to 2,448 rice PAC/BAC sequences. IRIS provides integrated information on genetic resources and Oryzabase provides information on genetic resources, genes, chromosome maps and general information on rice.

In 2000, with the completion of Arabidopsis genome sequencing, the *Arabidopsis* research community planned The *Arabidopsis* 2010 Program. The aim was to assign function to all the 25,000 *Arabidopsis* genes (Chory et al., 2000). Further, an International Rice Functional Genomics Consortium (IRFGC) has been set up with the goal of sharing knowledge, integrating the databases and functional genomics of rice (http://www.iris.irri.org/IRFGC/) hand in hand with the rice genome sequencing (Hirochika et al., 2004). IR64 deletion mutants developed at IRRI are accessible through the International Crop Information System database (ICIS), both phenotypic and molecular data related to the mutants are available at the site (Leung et al., 2001).

Besides the above, other mutant databases have also used the model plants *Arabidopsis* and rice whose sequence data is already available. The two major resources for *Arabidopsis* mutants generated through insertional mutagenesis are The Arabidopsis Biological Research Center (ABRC) and Nottingham Arabidopsis Stock Center (NASC) (Table 3). In fact, ABRC has the largest collection; it has more than 140,000 mutants generated through transposon and T-DNA tagging. Most of these have been contributed by the Salk institute (http://signal.salk.edu/tabout.html). Of the 140,000 mutants available, sequence information of flanking sites is available for 50,256 lines. If a researcher is not able to find a knockout in the gene of interest in these two databases for *Arabidopsis* then the other option would be to search in the The Arabidopsis Knockout facility (Krysan et al., 1999). Another database, which serves to integrate the data available from several projects on insertional mutagenesis, is the *Arabidopsis thaliana* insertion database (ATIDB; http://atidb.cshl.org/). Through this source more than 100,000 insertion lines can be accessed consisting of 196 defective Supressor/mutator (*dSM*), 6113 Supressor mutator (*SM*) lines, 119 activation tagging, 1586 gene trap, 1289 enhancer trap and 94,947 T-DNA lines (Pan et al., 2003). Users can search for T-DNA or transposon insertions in the sequence of interest and the stocks can be ordered directly through NASC and ABRC. To establish a T-DNA tagged population in which a T-DNA insertion can be found at a probability of ~99%, ~200,000 tagged lines of *Arabidopsis* and ~471,000 tagged lines of rice would be required (Jeon et al., 2000). For *Arabidopsis*, such a resource is now available (Table 3) where T-DNA tagging has been done on a genome-wide scale with more than 225,000 T-DNA inser-

tion lines available. Detailed analyses of 88,122 lines have revealed mutations in ~74% of *Arabidopsis* genes (Alonso et al., 2003). On the other hand, though a database is available for T-DNA tagging lines of rice, it is not saturated, representing less than 10% the number of lines required (Jeon et al., 2000). A total of 13,450 lines of rice are also available which can be used both for promoter trapping and activation tagging (Jeong et al., 2002). Chen et al. (2003) also characterized over 1000 T-DNA tags in rice genome. There are several other rice mutant resources developed by the international rice research community (Hirochika et al., 2004). A large resource of Mutator (*Mu*) insertions is also available for maize (May et al., 2003).

Several institutes and companies in Europe have formed consortia (European Consortia for Functional Genomics; see table 3) for functional genomics. GenoPlante-Info (GPI; http://genoplante-info.infobiogen.fr/); FLAG DB/FST database (Flanking sequenced tags; ~10,000 sequences flanking T-DNA insertion sites); CATMA (Complete *Arabidopsis* Transcriptome Micro-Array; 21,120 gene specific tags are available at the site); GPI EST database (EST's are available from *Arabidopsis*, rice, *Sorghum bicolor*, *Medicago truncatula*, *Zinnia elegans*, wheat, rapeseed, sunflower and pea) and The Plantgene database (collection of *Arabidopsis* full length cDNA's) and SAGE data (Samson et al., 2003).

Few databases have been described lately which are more specific, these include PlantsP and PlantsT functional genomics database which specifically provide information related to protein phosphorylation and membrane transport respectively (Tchieu et al., 2003). Both these databases not only provide sequence related information about protein kinases, phospahatases, and membrane transport proteins but also data on experimental functional genomics. One major database dedicated to stress genomics is the Stress Functional Genomics Consortium (http://stress-genomics.org/). It contains a collection of stress responsive ESTs from *Arabidopsis thaliana*, *Oryza sativa*, *Mesembryanthemum crystalinum*, *Hordeum vulgare*, *Selaginella* and *Dunaliella*. A collection of *Arabidopsis* mutants altered in stress signaling and global gene expression profiles of *Arabidopsis*, rice and ice plant under stress conditions can also be accessed through the site. In future, it would be desirable to integrate information spread over several databases.

7. CONCLUSIONS

Use of traditional approaches for studying gene function has given way to genome-wide approaches and as the sequencing data from more plants are becoming available, the interest is shifting to see what lies beyond the 'bases'. The task of identifying the function of each gene and their inter-relationships is not going to be easy unless more genome-wide approaches are employed. For instance, a large collection of insertional mutant plants freely available through several databases, microarrays and their modifications, proteome analysis and other techniques which have developed side by side to the large scale sequencing programs of model plants such as *Arabidopsis* and rice are now available. Expression profiling would give ready clues only about the

Table 4. ***Some databases for sequenced flanking insertion sites***

Source	Resource	Website	Reference
Nottingham *Arabidopsis* Stock Center (NASC), England	~500 Ds insertion flanking sequences *(Arabidopsis)*	http://nasc.nott.ac.uk/ima.html	Wilson et al. (1999).
European Consortia	60,000 sequenced insertion sites *(Arabidopsis)*	www.jic.bbsrc.ac.uk/science/cdb/exotic/	Hilson et al. (2003).
SALK Institute, USA	~94,947 T-DNA Flanking sequences *(Arabidopsis)*	http://signal.salk.edu/cgi-bin/tdnaexpress	Pan et al. (2003).
Torrey Mesa Research Institute (TMRI) USA	~100,000 T-DNA flanking sequences *(Arabidopsis)*	http://www.tmri.org/pages/collaborations/garlic_files/	Pan et al. (2003).
Cold Spring Harbor Laboratory, USA	~26,800 Ds, gene trap and enhancer trap lines *(Arabidopsis)*	http://genetrap.cshl.org/	Pan et al. (2003).
John Innes Centre, England	~13,000 DSpm and Ds gene trap lines *(Arabidpsis)*	http://atidb.cshl.org/	Pan et al. (2003).
Arabidopsis Biological Research Center, USA	50,256 T-DNA flanking sequences *(Arabidopsis)*	http://arabidopsis.org/abrc/	Rhee et al. (2003).

expression levels of genes in response to different stresses, which can be used to characterize mutants. Mutant analysis in turn will give more definite knowledge of each gene function but even in case of mutant analysis the phenotype is not always evident and requires complementation studies. Hence, transgenics would be needed before function can be established or a new functional allele tested. The key point is that no single technique will help identify gene function but the focus should be on an integrated approach. The information obtained from gene discovery and expression profiling should be complemented with a systematic study of tagged mutant collections. Complementation and over-expression studies will help identify the key players of stress response and also their inter-relationships (Fig. 2). The genes identified through such effort would not only reveal gene function but would also identify the particular combination of genes. Pyramiding of such genes will help develop 'the model stress tolerant crop' which is of course the ultimate aim of these efforts.

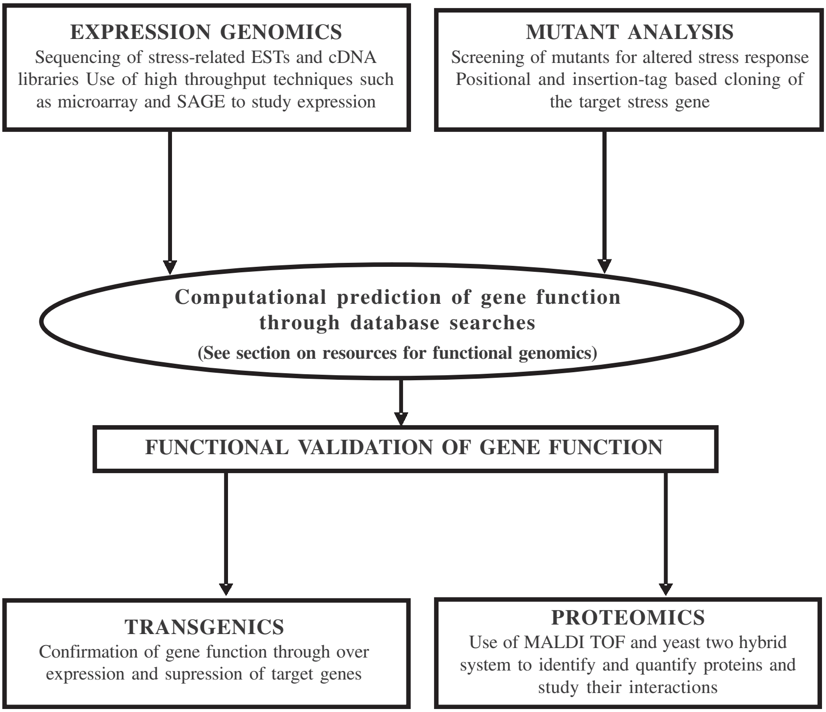

Figure 2. *A step-by-step functional genomics strategy to study stress tolerance in plants*

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